Perivascular Macrophages in Neuroinflammation, the role of the Scavenger Receptor CD163

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Perivascular Macrophages in Neuroinflammation, the role of the Scavenger Receptor CD163

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Paranimfen:	Elske Stellingwerff Beintema Frederique J. Ummels

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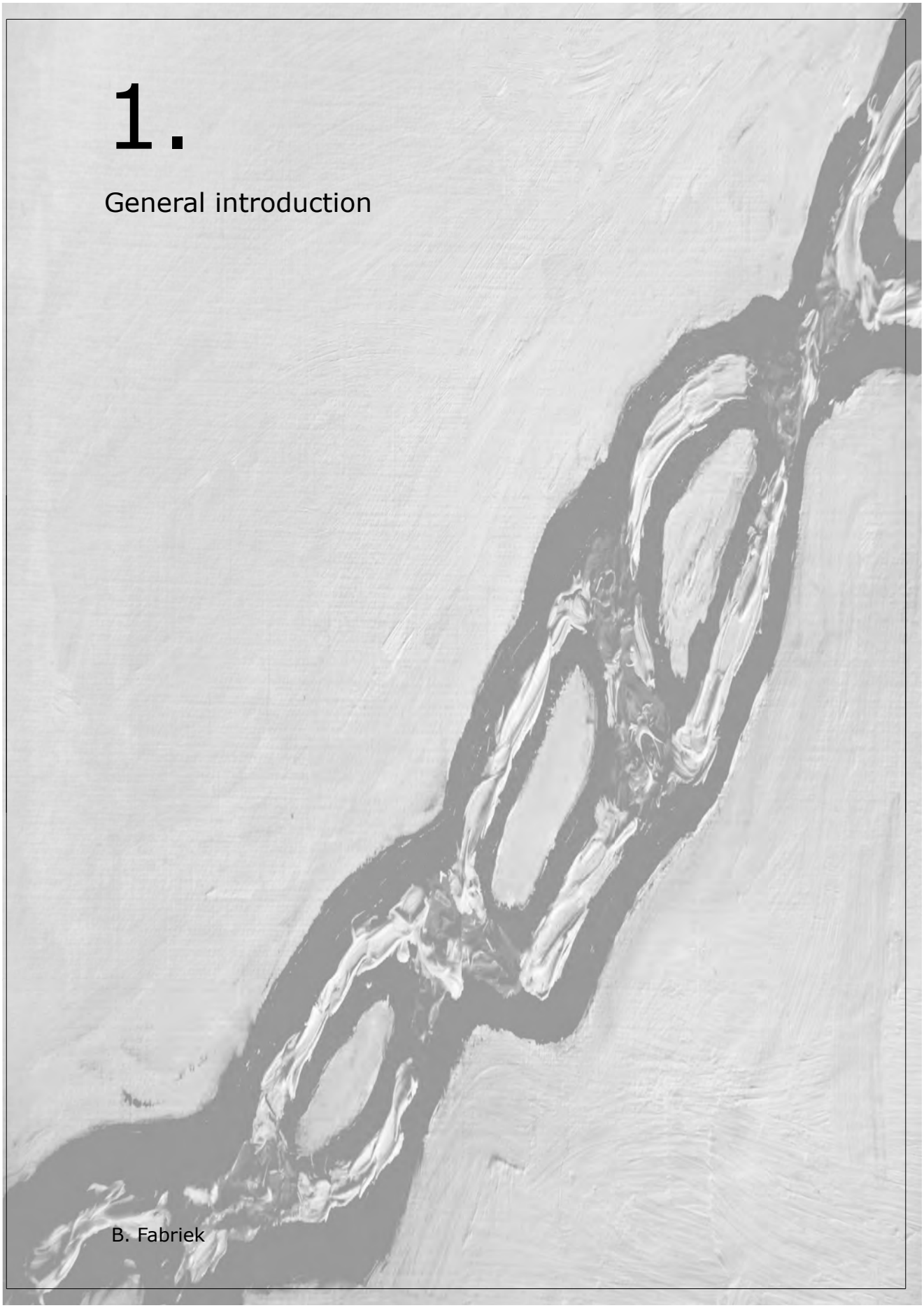
Abbreviations

APC	antigen presenting cell
BBB	blood brain barrier
BSA	bovine serum albumin
CD	cluster of differentiation
CFU	colony forming units
CHO	Chinese hamster ovary
CLN	cervical lymph nodes
CNS	central nervous system
CSF	cerebrospinal fluid
COX-2	cyclo-oxygenase-2
DAB	3.3'-diaminobenzidine tetrahychloride
DC	dendritic cell
DC-SIGN	dendritic cell-specific ICAM- 3 grabbing nonintegrin
DEX	dexamethasone
DMSO	dimethylsulfoxide
EAE	experimental autoimmune encephalomyelitis
EB	erythroblast
EDSS	expanded disability status scale
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EPO	erythropoëtine
FACS	fluorescent activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GC	glucocorticosteroids
Hb	hemoglobin
HC	healthy control
HIV	human immunodeficiency virus
HO	heme-oxygenase
Hp	haptoglobin
IFN	interferon
Ig	immunoglobulin
IL	interleukin
iv	intravenous
LCA	leukocyte common antigen
LTA	lipoteichoic acid
LPS	lipopolysaccharide
mAb	monoclonal antibody
MBP	myelin basic protein
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MM	meningeal macrophage(s)
MMP	matrix metalloproteinase
MOG	myelin oligodendrocyte glycoprotein
MP	methylprednisolone
MR	mannose receptor
MS	multiple sclerosis
PAMP	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PLP	proteolipid protein
PO	peroxidase
PP	primary progressive
QPCR	quantitative polymerase chain reaction
PVM	perivascular macrophage(s)
RA	rheumatoid arthritis
RR	relapsing remitting
RT	room temperature
SCD163	soluble CD163
SD	standard deviation
SIV	simian immunodeficiency virus encephalitis
SMA	smooth muscle actin
SP	secondary progressive
SR	scavenger receptor
SRCR	scavenger receptor cysteine-rich
TBS	tris buffered saline
TIMP	tissue inhibitor of MMP
TLR	toll-like receptor
TNF	tumor necrosis factor
USgFNAC	ultrasound-guided fine needle aspiration cytology
WT	wild type

1.

General introduction



B. Fabriek

1. The Central Nervous System: an immune privilege site?

The brain has been considered to be an immune privileged site for a long time. The first study to suggest this immune privilege was of Peter Medawar (Medawar, 1948), who demonstrated that foreign skin transplanted into the brain was tolerated over an extended period of time. We now know that this immune privilege is a result of active as well as passive processes. Several features of the central nervous system (CNS) are important to maintain this immune privileged state. For example, the CNS is devoid of lymphatic vessels, lacks major histocompatibility complex (MHC) I expression in the parenchyma, and does not contain large amounts of MHC class II expressing antigen-presenting cells (APC). However, the latter is currently under debate and will be further discussed in this thesis.

The brain is protected from invaders by the endothelial blood-brain barrier (BBB) and the epithelial blood-cerebrospinal fluid barrier. The BBB relies on the presence of tight junctions between endothelial cells. Tight junctions exclude passage of cells and macromolecules between the brain and the blood circulation. To maintain the BBB related properties, endothelial cells require a constant communication with glial cells (Engelhardt and Ransohoff, 2005).

On the abluminal side of the endothelium lies a continuous basement membrane composed of a mixture of substances, including type IV collagen, heparan sulphate proteoglycan, laminin, and entactin (Kalluri, 2003; Van Horssen et al., 2005). Astrocytes project their end-feet tightly onto the endothelial cells and cover the total abluminal surface of the brain capillaries (Broadwell et al., 1994). Pericytes and perivascular macrophages (PVM; discussed in more detail in Chapter 2) are completely surrounded by the basement membrane and pericytes are frequently seen extending their processes around the blood vessel.

However, the BBB is not an absolute barrier, there are mechanisms by which cells and molecules necessary for proper CNS function can traverse this barrier. PVM produce pro-inflammatory cytokines, such as IL-1β during inflammatory responses in the CNS (Bauer et al., 1993; Angelov et al., 1998b) and may thereby promote adhesion molecule expression by endothelial cells, stimulating leukocyte infiltration into the CNS.

1.1. Immune Surveillance

In normal, non-pathological conditions the number of naive or activated T lymphocytes traversing the BBB is low (Williams and Hickey, 1995; Hickey, 1999; Brabb et al., 2000). However, this is still believed to contribute to the general immune surveillance of the brain. When there is a strong immunological reaction in the body (even if the CNS is not primarily involved) the amount of lymphocytes passing over the BBB increases (Hickey and Kimura, 1987). The role played by the lymphocytes entering the CNS can best be described as antigen seeking (Hickey et al., 1991). Moreover, activated T lymphocytes (Wekerle et al., 1987) have an increased ability to cross the BBB compared to naïve T lymphocytes (Brabb et al., 2000). These lymphocytes are

primed within the lymphoid organs and recirculate in the peripheral blood, looking for their antigen presented by APC within tissues.

In the brain there are several populations of cells of the mononuclear phagocyte lineage that could serve as APC. In the brain parenchyma the most numerous potential APC are the infiltrated macrophages, the microglia, and PVM. Typical MHC class II positive, OX62+ dendritic cells can only be found in the meninges and choroid plexus and not in the brain parenchyma (Matyszak and Perry, 1996; McMenamin, 1999), at least in non-pathological conditions.

During inflammatory reactions in the CNS, large numbers of lymphocytes and monocytes are recruited from the circulation; upon entering the parenchyma monocytes rapidly acquire a typical macrophage morphology and phenotype. In contrast to infiltrating macrophages or PVM, microglia have a resting or down-regulated phenotype and do not normally express high levels of MHC class I and II (Perry, 1994). Microglia have extensive cell processes and are located throughout the parenchyma. When microglia cells become activated they become more rounded, upregulate MHC class I and II and phagocytic receptors, start producing tumor necrosis factor (TNF)-α, and display an extended lysosomal compartment (Perry et al., 1993; Bauer et al., 1996). This suggests that these cells may perhaps contribute to antigen presentation during later stages of brain inflammation. However, at the same time these changes also make it more difficult to distinguish activated microglia from infiltrating macrophages in inflammatory brain lesions.

In addition to the microglia and infiltrating macrophages there are two other subpopulations of macrophages within the CNS, the PVM and meningeal macrophages (MM). These resident macrophages have a distinct phenotype (Hayes et al., 1987; Graeber et al., 1989; Kida et al., 1993; Angelov et al., 1998a) and constitutively express MHC class II (Hickey and Kimura 1988). PVM may play a pivotal role in the recognition and uptake of pathogens and their degradation products present in the bloodstream or the brain parenchyma (Kida et al., 1993; Mato et al., 1996) and as such may contribute significantly to the initiation of both innate as well as adaptive immune responses in the brain.

The PVM express a variety of receptors enabling pathogen recognition and uptake, including members of the scavenger receptor and C-type lectin families. These receptors have a relatively broad ligand binding specificity (Resnick et al., 1994; Krieger, 1997) and can mediate the recognition and uptake of a variety of pathogens, including viruses, bacteria and fungi (discussed in more detail in Chapter 2). One of these receptors is the scavenger receptor CD163 (introduced in more detail in Chapter 3), which will be discussed more extensively in the following chapters of this thesis.

Ligation of the PVM-associated scavenger receptor CD163 with its ligand, hemoglobin-haptoglobin complexes, or with the monoclonal antibody EDHu-1 results in the production of pro- and anti-inflammatory cytokines (Van den Heuvel et al., 1999; Kristiansen et al., 2001; Ritter et al., 2001; Moestrup and Moller, 2004). This is consistent with a role for PVM in initiation of a local (CNS) immune response, upon contact with proteins derived from the blood circulation or the brain parenchyma.

1.2. Drainage of the CNS to the peripheral lymphoid tissues

As mentioned before, the CNS is an immune privileged site maintained due to the presence of a BBB, constitutively low T cell trafficking and low MHC class I and II expression, but also by the lack of lymphatic drainage pathways. It has been suggested that brain antigens reach the peripheral lymphoid system, either via drainage through the nasal lymphatics, or via drainage through the ventricular system to the choroid plexus (Weller et al., 1992; Zhang et al., 1992) (Figure 1).

Cerebrospinal fluid (CSF) circulates within the brain through the ventricles and is delivered to the basal cisterns via the foramen of Magendie and the Foramina of Luschka (both are openings in the fourth ventricle), where it ends into the subarachnoid space (Harling-Berg et al., 1999). Interstitial brain fluid (ISF) also drains into the subarachnoid space, which it reaches through the Virchow-Robin spaces. It has long been known that substances introduced into the brain parenchyma can be transported from the subarachnoid space into blood and into the cervical lymph nodes (CLN) (Figure 1) (Zhang et al., 1992; Cserr et al., 1992; Kida et al., 1993).

The CLN are the first draining site of the brain and may thus generally be the first site of encounter between brain-derived antigens and naive T lymphocytes (Weller, 1998). Previously, it has been shown that injecting a soluble antigen into the grey matter of the rat brain resulted in specific antibody formation in the CLN (Harling-Berg et al., 1989). Furthermore, the removal of the CLN from rats with experimental auto-immune encephalomyelitis (EAE) reduced the severity of cerebral EAE by 40%, which has suggested a significant contribution for the CLN to T cell priming and/or maintenance of the specific T cell response (Phillips et al., 1997). On the other hand, the CLN microenvironment may also preferentially (i.e. in comparison to peripheral lymph nodes) support the development of specific tolerance, as has been demonstrated after CLN removal, which abrogated the induction of tolerogenic regulatory T cells (Wolvers et al., 1999; Unger et al., 2003).

The mechanism(s) of antigen transport from the CNS to the CLN are essentially unknown. Understanding of these mechanisms may, as suggested above, be relevant for induction and/or maintenance of autoimmunity in the CNS. Antigen transport in the CNS could either be cell-mediated or cell-independent, or both. As indicated above PVM are particularly effective scavengers of the perivascular space in the CNS (Kida et al., 1993; Mato et al., 1996; Angelov et al., 1996) and may thus play a major role in antigen uptake, but the precise routes of the ingested particles and antigens remains unclear. Some authors suggest that the perivascular cells, which have ingested particles remain immobile in the perivascular space and that only soluble molecules travel into the fluid phase of the perivascular space (Zhang et al., 1992), while others suggest that the PVM travel directly to the CLN for presentation of their antigen (De Vos et al., 2002; Karman et al., 2004).

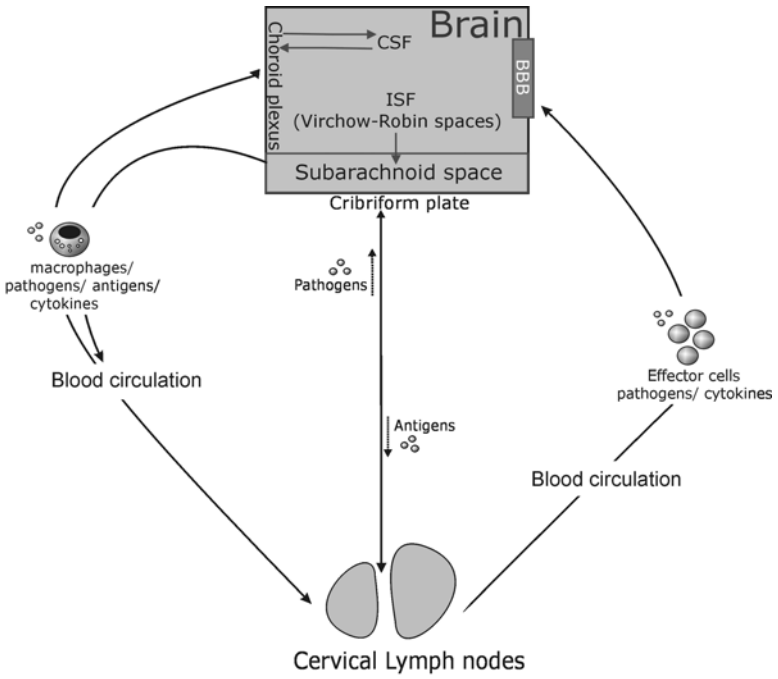


Figure 1: *Schematic representation of afferent and efferent CNS pathways*.

When an inflammation in the brain occurs, brain-derived antigens (e.g. neuronal antigens, myelin proteins) drain to the CLN (or spleen) either via the lymphatics of the nasal mucosa or via the blood circulation, transported as particles or within cells. Effector cells from the CLN are then primed and redirected to the brain parenchyma. Also pathogens can enter the brain via the bloodstream or through the nasopharynx (discussed in paragraph 1.2.).

2. Inflammatory reactions in the brain

During various pathological conditions in the CNS, such as viral or bacterial infections, ischemia or chronic inflammatory diseases, leukocytes pass the BBB and accumulate within the perivascular spaces. The location and composition of these brain infiltrates differs with the type disease. In EAE, the animal model for Multiple Sclerosis (MS), the perivascular and parenchymal infiltrates are devoid of neutrophils. In bacterial infections, such as meningitis and encephalitis, there is a massive influx of neutrophils. Infection of the meninges, the membranes that surround the brain and spinal cord, is referred to as meningitis, whereas infection of the brain parenchyma is referred to as encephalitis.

2.1 Multiple Sclerosis

MS is a chronic inflammatory demyelinating disease of the CNS characterized by the invasion of the brain by T cells and macrophages through the BBB, causing damage to myelin sheaths and axons (Lassmann, 1998). MS is the most common chronic

neurological disease of young adults, with the onset of the disease between age 20 and 40. Women are more often affected than men, in a ratio of approximately 2:1. The prevalence of MS is approximately 1 in 1000 for the Northern part of Europe and the USA, and 1 in 20,000 in equatorial areas. Several hypotheses have been postulated for the etiology of MS, altogether it has been suggested that both genetic and environmental factors contribute to the susceptibility for this disease (Rosati, 2001).

MS is characterized by the formation of multiple sclerotic lesions (MS-plaques) in the CNS. This lesion formation results in loss of motor and/or sensory function. The first symptoms may include vision-loss, motor function disturbances and sensory abnormalities. Since there is no typical pattern of symptoms or neurological signs for MS, magnetic resonance imaging (MRI) techniques are important for diagnosis of MS and the detection of new lesions during the disease process (McDonald et al., 2001).

Different progression forms of MS exist, each with a different clinical course. Approximately 70% of the patients have the relapsing remitting (RR) form of MS, characterized by a series of attacks (relapses) resulting in a temporary increase in disability from which the patients recover partially or completely (remission). In time, the majority of the RR MS patients will gradually change to a more progressive form of disease (secondary progressive, SP). About 10-20% of all MS patients show a primary progressive (PP) disease course from onset. The progressive form of the disease lacks the acute relapses but is characterized by a gradual clinical decline. In a proportion of the patients (approximately 15-20%) the disease is progressive with presence of superimposed exacerbations in the course of time. Patients with the so-called benign form of MS completely recover after one or two relapses and show modest or no progression of the disease (Lublin and Reingold, 1996).

For the individual patient the clinical course of MS is unpredictable, therefore it is of great importance that diagnostic tools are developed to predict the clinical course of the disease and to evaluate the effects of therapy.

2.1.1. Pathology and lesion staging

The pathological hallmarks of MS in the brain and spinal cord are inflammation, demyelination, axonal loss and gliosis (Charcot, 1868). MS lesions can be classified as (p)reactive, active, chronic active and chronic inactive (Van der Valk and De Groot, 2000; De Groot et al., 2001). In (p)reactive lesions there are discrete white matter abnormalities, such as clusters of strongly MHC class II and $CD45^+$ microglial cells, and occasional perivascular infiltrates, but no demyelination. Active, demyelinating lesions are characterized by the presence of macrophages containing myelin degradation products (foamy macrophages), and infiltrating T lymphocytes. The presence of myelin proteins in these macrophages indicates ongoing demyelination (Bruck et al., 1995; Van der Goes et al., 2005). The astrocytes within these lesions are activated and hypertrophic. Chronic active demyelinating lesions contain a hypocellular, demyelinated centre with foamy macrophages located at the rim of the lesion. Moreover, oligodendrocytes are reduced in number and lymphocytes are present in the perivascular areas. Chronic inactive lesions have a demyelinated area with widened extracellular spaces and gliotic scar tissue without cellular infiltrates. Besides

demyelination also remyelination is present in some lesions, these are so-called shadow plaques characterized by oligodendrocyte precursors, astrogliosis and myelinating axons (Lassmann, 1998; Van der Valk and De Groot, 2000; De Groot et al., 2001).

2.1.2. Cytokines in MS

Cytokines are low-molecular weight immunoregulatory proteins that are produced by a variety of cells in a variety of tissues. Cytokines can have pro- and anti-inflammatory activities and thereby regulate immune responses and inflammatory reactions. Examples of pro-inflammatory cytokines are interferon (IFN)-γ, interleukin (IL)-1, IL-6, IL-12, IL-23 and TNF-α and of anti-inflammatory cytokines, IL-10, IL-4, IL-5 and IL-13 and transforming growth factor (TGF)-β. An optimal balance in cytokine production and T cell responses is necessary for an adequate immune response, whereas a disturbance in this balance may lead to disease.

Upregulation of several pro- and anti- inflammatory cytokines has been observed in MS, although cytokine abnormalities found in MS are not disease-specific and can also be found in other inflammatory CNS diseases. Moreover, there is a relation between cytokine levels and disease activity in MS, since TNF-α and IFN-γ are correlated to clinical relapses and TGF-β, IL-12p40, and IL-10 are related to the remission phase of the disease (Link et al., 1994; Rieckmann et al., 1994; Navikas and Link, 1996; Boxel-Dezaire et al., 1999). Pro-inflammatory cytokines are suggested to participate in MS pathology whereas anti-inflammatory cytokines, such as IL-4, IL-10 and TGF-β could reflect immunoregulatory mechanisms that are initiated after disease exacerbations and are important for disease resolution, or in the case of EAE disease prevention (Sospedra and Martin, 2005).

2.1.3. Treatment

The current treatment of MS has relied on immunosuppressive, immunomodulatory drugs and combination therapies, which aim to reduce relapse frequency and prevent disability increase (Rudick et al., 1997; Compston and Coles, 2002). Reduction of the relapse rate and diminishing relapse severity is best achieved by the use of β-interferons. The mechanism by which β-interferons exert their therapeutic effects is very complex and includes antagonizing effects on the production of pro-inflammatory cytokines, downregulation of MHC class II antigens and attenuation of BBB disruption (Hall et al., 1997; Compston and Coles, 2002). Other substances that reduce the relapse rate and the increase of disability are glatiramer acetate, azathioprine, and mitoxantrone that all have similar efficacy as β-interferons (Paty and Li, 1993; Johnson et al., 1995; Perini and Gallo, 2003).

Disability as a consequence of acute relapses is treated with a high dose of glucocorticosteroids (GC) given as a treatment regimen of 3 or 5 days of intravenous methylprednisolone (iv-MP). Clinically short-term administration of MP results in reduced severity of symptoms and promotes faster recovery of clinical attacks (Pozzilli et al., 2004). GC treatment prevents the disruption of the BBB, decreases the production of pro-inflammatory cytokines, decreases secretion of immunoglobulins,

and inhibits the expression of MHC class II on macrophages and microglia (Barnes and Adcock, 1993; Andersson and Goodkin, 1998; Pozzilli et al., 2004). Unfortunately, not all patients respond equally well to GC. Strikingly, up to one third of the patients with an inflammatory disease are non-responsive to GC therapy (Norman and Hearing, 2002). The underlying mechanism behind this non-responsiveness is to date yet unknown.

Overall, it can be assumed that the anti-inflammatory effects of GC and β-interferons are quite similar since they both have effects on BBB permeability and on restoration of the imbalance in cytokine production. New therapies are currently being developed to offer better treatment. These therapies target: the BBB (via the inhibition of leukocyte adhesion, metalloproteinase and chemokines inhibitors), immune suppressive properties (anti-MHC class II antibodies, blockers of costimulatory signals, interleukin inhibitors), and neuroprotective properties (statins, minocycline, oxidative stress inhibition) (Noseworthy, 2003; Polman and Uitdehaag, 2003).

2.1.4. Animal Models for MS

Most animal models for MS are induced by immunization with myelin proteins together with complete Freund's adjuvant. The EAE model has been studied extensively and clinical and histopathological similarities to MS, have been found (Swanborg, 1995). However, in contrast to MS, EAE is not a spontaneous autoimmune disease. In genetically susceptible animals EAE requires either direct immunization with myelin antigen components, or adoptive transfer of anti-myelin specific $CD4^+$ T cells (Behi et al., 2005). Considerable variations in the susceptibility of various animal strains and species have been described. These variations are largely due to differences in genetic background and in particular to the MHC haplotypes of the animals (Petry et al., 2000). Depending on the immunization, the disease pattern may vary from a monophasic type of disease (acute and transfer EAE) to a demyelinating and relapsing remitting form of disease (chronic EAE). The clinical symptoms that arise start with loss of tail tonus, and progresses to hind limb paralysis (Willenborg, 1979).

Current understanding of EAE pathophysiological processes focuses on T cells, specific for myelin proteins such as myelin basic protein (MBP), proteolipid protein (PLP), or myelin oligodendrocyte glycoprotein (MOG), which are activated in the periphery and subsequently infiltrate the CNS, accompanied by a massive influx of monocytes, and cause autoimmune inflammation leading to paralysis. EAE has provided extensive insight into neuroinflammatory processes; however, none of the EAE models exactly simulates the disease course of MS. Thus it must be stated that translation of data obtained from EAE models to MS patients should be done with caution (Sriram and Steiner, 2005).

2.2. Infection of the brain

The brain is protected from bacterial invasion by the skull, the dura mater, the arachnoid membrane, the pia mater, and the glia limitans. Bacteria can penetrate into the brain if there is a break in the continuity of these protective layers, which can be caused by congenital defects or trauma. On the other hand, bacteria can also spread

into the brain from the nasopharynx, where they gain access to the bloodstream and are spilled into the CSF through the leaky capillaries of the choroid plexus. When the infection is spread into the CSF, which surrounds the brain and spinal cord, it can affect the nerves and travel to the brain and/or surrounding meninges. The subsequently occurring swelling can harm or destroy nerve cells and cause bleeding in the brain. The inflammation can produce a wide range of symptoms, including fever, a stiff neck, headache, or confusion, and in extreme cases, can cause brain damage, stroke, seizures, or even death. Worldwide the occurrence of meningitis is 1-2 million cases each year, depending on the causative microorganism the mortality can be as high as 20% (Lahrtz et al., 1998; Nau and Bruck, 2002). Meningitis is most often caused by a viral or bacterial infection. Viral meningitis is mainly caused by enteroviruses (Lahrtz et al, 1998), whereas the main cause of bacterial meningitis is infection with *Haemophilus influenzae*, *Neisseria meningitidis* or *Streptoccocus pneumoniae*.

The initial development of meningitis occurs in several steps. First, bacteria colonize the nasopharynx, followed by a local invasion of the mucosal epithelium. Once the mucosal barrier is crossed, bacteria gain free access to the bloodstream. Somehow bacteria are then capable to adhere to the endothelial cells of the brain vessels, thereby increasing the BBB permeability and facilitation of the meningeal invasion (van Furth et al., 1996; Tauber and Moser, 1999). Of relevance, the PVM and MM have been shown to play a protective role during bacterial meningitis, suggesting that the primary action of these macrophages is to facilitate the influx of leukocytes at the BBB (Polfliet et al., 2001).

A more important role for PVM have been suggested in HIV encephalitis, where the PVM appear to be the primary cells that get infected (Williams et al., 2001; Kim et al., 2003). The PVM have been suggested to function as so-called Trojan horses for the virus, since virus-infected and infiltrating monocytes traffic to the CNS from the blood to become PVM, and thereby potentially cause the BBB damage and neuronal injury (Kim et al., 2006).

3. Outline of this thesis

Clearly, studying the cellular and molecular mechanisms underlying inflammation in the brain is of central importance for the understanding of brain pathology. Moreover, this knowledge may form the basis for new treatment strategies.

In this thesis we have studied the role of the PVM and the PVM-associated scavenger receptor CD163 in the development of CNS inflammation, in particular MS.

In **Chapter 2** the potential role of the PVM within the brain is reviewed in more detail. The possible function(s) of the macrophage scavenger receptor CD163, which is prominently expressed on PVM and may also play a role in the regulation of CNS inflammation, is reviewed in **Chapter 3**.

In **Chapter 4** we investigated the expression of CD163 in the brains of healthy controls (HC) and MS patients. Furthermore, we examined if $CD163^+$ PVM within the human CNS express receptors critical for antigen recognition and presentation, which would support the idea that PVM act as local APC of the brain and induce secondary T cell activation. In **Chapter 5** we address the question whether brain-derived myelin

antigens are present within macrophages in the CLN of MS patients, since the CLN are a putative site of systemic presentation of brain antigens and priming of T lymphocytes.

An increase in soluble CD163 levels was observed by others in a variety of diseases, where macrophages are involved in the pathogenesis of the disease. Since this also holds true for MS, we examined the levels of soluble CD163 in the plasma of MS patients and HC, in order to investigate if soluble CD163 would be a useful biomarker for macrophage activity in MS patients **(Chapter 6).** Membrane CD163 is known to be upregulated by GC *in vitro*, whether this also occurs after *in vivo* administration of GC was the question we asked in **Chapter 7**. In addition, we wondered if GC induced CD163 expression on monocytes *in vitro* could predict the therapy responsiveness of MS patients *in vivo*.

In the last three chapters of the thesis we address several molecular, regulatory and functional aspects of CD163 in more detail, in particular aspects involved in cell-cell interaction and cell-pathogen interaction (**Chapters 8-10**). In **Chapter 8** we study the expression and regulation of rat CD163, in order to be able to correlate our findings on PVM in rat (EAE) studies to those in humans, including MS patients. In **Chapter 9** we investigate the role of CD163 in cell-cell interaction, in particular we study whether CD163 might function as a receptor for erythroblasts, as is indicated but not proven in previous studies. Whether CD163 might also function as bacterial binding receptor is addressed in **Chapter 10**.

Finally, in **Chapter 11** the results are summarized and the role of the CD163-expressing PVM during CNS inflammation is discussed.

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2.

Cerebral perivascular macrophages and the blood-brain barrier

Book chapter, Chapter 12; The blood-brain barrier and its microenvironment (editors: A. Pratt and H.E. de Vries), Taylor & Francis Group, 2005: 295-308

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1. Perivascular macrophages in the central nervous system

The blood-brain barrier (BBB) is a composite structure resulting from the contribution of several different cell types and their extracellular matrix. Its major constituents are the endothelial cells, which undergo barrier specialization as a result of their interaction with the foot processes of astrocytes (glia limitans). The perivascular space, between these two cell types, represents a tightly regulated microenvironment. It harbours 3 cell types: perivascular macrophages (PVM), pericytes and smooth muscle cells. This chapter deals with PVM, which are distinct from their myeloid counterparts both within (microglia) and outside (monocytes) the brain parenchyma. By virtue of their strategic location at the BBB, PVM lend themselves to a variety of potentially important functions in both health and disease. Examples of such functions include recognition, phagocytosis, degradation, and transport of pathogens, antigen presentation to T lymphocytes, and production of immune regulatory mediators. Furthermore, some aspects of the phenotype of PVM suggest that they contribute to the maintenance of the BBB.

1.1. Morphological characteristics of perivascular macrophages

PVM are the second most common type of resident tissue macrophages in the central nervous system (CNS), after the microglia. PVM are readily recognized by their location, between the endothelial basement membrane and the glia limitans in the perivascular space of arteries, medium sized vessels, and capillaries (Graeber et al., 1992; Mercier et al., 2002). PVM are often missed in routine stainings due to their relative infrequency. They have an elongated bipolar morphology and contain lysosomes in their cytoplasm (Figure 1). The nomenclature of these cells is somewhat confusing. Throughout the years, PVM as we know them, were called fluorescent granular perithelial cells (Mato et al., 1986), perivascular microglia (Hickey and Kimura, 1988), pericytes (Broadwell and Salcman, 1981; Hickey and Kimura, 1987), ED2 positive perivascular cells (Graeber et al., 1989), or perivascular monocytes (Vass et al., 1986; Lassmann et al., 1991).

PVM are different from pericytes, which are fibromyocytic cells within the blood vessel wall, yet some reports do not distinguish between PVM and pericytes (Balabanov et al., 1996). Pericytes are, like PVM, a cellular constituent of the BBB, but lie completely enclosed within the vascular basement membrane on the abluminal side of the endothelium of small blood vessels (Figure 2 and Figure 1, Chapter 4) (Graeber et al., 1992). Although pericytes have an oval to elongated cell body similar to PVM, they are more flattened. Pericytes possess branching processes that encircle the blood vessel (Hickey et al., 1992; Kida et al., 1993). They are thought to provide vasodynamic capacity and structural support to the microvasculature of the brain (Balabanov and Dore-Duffy, 1998).

PVM are distinct from microglia, the other resident mononuclear phagocyte population in the CNS. Microglial cells are ramified cells that occur in the CNS parenchyma with their processes arranged longitudinally along nerve fibre tracts in the white matter or in a more stellate pattern in the grey matter.

Figure 1: *Perivascular macrophage network.*

A. The blood vessel wall consists of endothelial (End) and pericyte/smooth muscle (Per/SMC) layers. In addition, this blood vessel (BV) shows a continuous macrophage (Mac) layer. The macrophages contact one another by their processes (arrow heads) to form a network. Basal lamina (BL) projection encloses parenchymal cell processes (arrow). **Top inset:** 2x magnification of the area indicated by a long arrow. **Bottom inset:** 3x magnification of a zone showing the spreading out of the basal lamina in the blood vessel. One can distinguish the first perivascular basal lamina (covering the macrophages, arrowhead) from the basal lamina projections (double arrowhead) enclosing processes of parenchymal cells. **B.** A capillary showing the nucleus of a macrophage and a portion of another macrophage containing a large lysosome (lys). Both cells are covered by the perivascular basal lamina (arrows). **C.** A small capillary showing no perivascular cell. **D.** Location of the images A, B, and C. CC: corpus callosum, Cpu: caudate putamen; Ep. Ependyma; LV: lateral ventricle. Scale bars = 5 μm in A, 1 μm in B and C, and 50 μm in D. This figure was prepared and previously published by Fredric Mercier (Mercier et al., 2002), who kindly gave his permission to incorporate this figure in the current review.

Unlike PVM, microglia dramatically change their morphology upon activation: their processes become shorter and stouter and their cell bodies more rounded. Indeed in many circumstances microglia cannot be distinguished from blood-derived macrophages (Streit et al., 1988). It was demonstrated by means of bone marrow chimeras that both PVM and microglia are derived from blood monocytes (Hickey et al., 1992). Microglial cells arrive before birth and persist for long periods with little repopulation from the bone marrow (De Groot et al., 1992). PVM, on the contrary, are replaced every 2 or 3 months by monocytes immigrating from the bone marrow (Hickey and Kimura, 1988; Hickey et al., 1992). The fate of PVM upon their replacement is so far unknown: they might die by apoptosis or migrate to other sites.

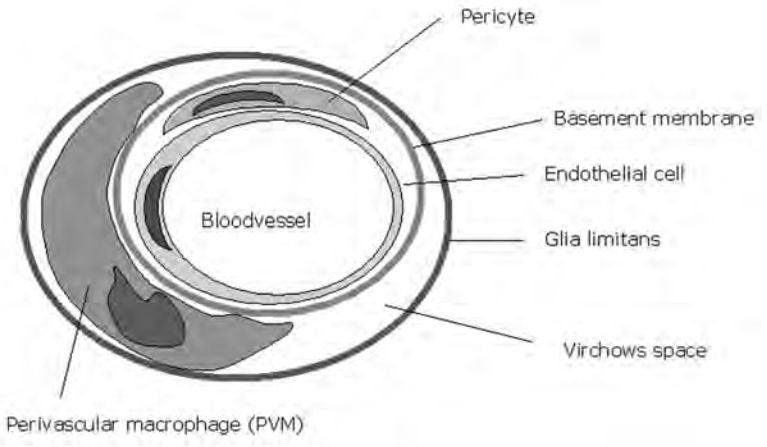

Figure 2*: This figure shows a schematic drawing of the spatial interrelationships between the various structures in and around the blood vessel wall.*

1.2. Cellular markers specific for PVM

Apart from morphology, PVM express cellular markers that distinguish them from microglial cells and pericytes (summarized in Table 1) (Angelov et al., 1998b). In rat and human CNS, PVM can typically be recognized by antibodies against CD163 (rat: mAb ED2 (Graeber et al., 1989) and Chapter 8, human: mAb EDHu-1 (Van den Heuvel et al., 1999) and Chapter 4), which is not expressed by any other cell in the CNS. In mouse and human CNS, the mannose receptor is specific for PVM (Galea et al., 2005) (Chapter 4). Furthermore, PVM have higher levels of major histocompatibility complex (MHC) I and II than microglial cells (Hickey and Kimura, 1987) and are $CD45^{high}$ (leukocyte common antigen; LCA), whereas microglial cells are $CD45^{low}$. The latter appears to be the case in both macaques (Williams et al., 2001b) and rodents (Ford et al., 1995). In macaques, CD14 is only expressed by PVM and not microglia and has been used to distinguish between these two populations in simian immunodeficiency virus encephalitis (Williams et al., 2001b). In humans, however, CD14 and CD45 are not discriminatory markers in pathology such as human immunodeficiency virus (HIV)-1 encephalitis (Cosenza et al.,

2002). Moreover, in the latter study, CD45 was not specific for PVM in control brain either (Cosenza et al., 2002).

Pericytes can be distinguished from perivascular cells by the presence of smooth muscle actin (SMA) (Herman and D'Amore, 1985) and their lack of macrophage markers. Pericytes, not PVM, express receptors for vasoactive mediators like catecholamines, endothelin-1, vasoactive intestinal peptide, vasopressin and angiotensin II, which suggests that pericytes might be involved in cerebrovascular tone and blood flow regulation (Balabanov and Dore-Duffy, 1998).

Apart from their morphology and expression of cellular markers, the PVM's phagocytic capacity can be used as a marker since PVM are the only constitutively phagocytic cells in the absence of inflammation. This may circumvent the possibility that not all PVM express conventional markers such as CD163, which might result in an underestimation of their number. Various substrates have been used to this end including intrastriatally injected Indian ink (Kida et al., 1993) as well as intracerebroventricularly injected horseradish peroxidase (Walther et al., 2001), fluorescent dextrans (Bechmann et al., 2001), FluoroGold (Angelov et al., 1998a), the green fluorescent cell tracker CFDA (Bechmann et al., 2001), DiI-labeled liposomes (Polfliet et al., 2001a), and fluorescent microspheres (authors' experience). A drawback of this method is that it cannot be used on human specimens to identify PVM.

2. Phenotype and function of PVM in the healthy CNS

There are very few studies that functionally interrogate the role of PVM, their function being largely inferred from their surface molecule expression in descriptive studies. Although it is fair to assume function complementary to a particular molecule with a known activity, this approach has some caveats: it does not take into account possible unknown functions of the molecule in question or its dependence on, cooperativity with, or inhibition by other unknown molecules. Moreover, this approach is unable to properly assess the extent of the PVM's contribution to the function in question and whether it is redundant or not.

On the other hand, functional studies address these issues directly by manipulating the PVM population selectively. Several tools have been used to this end, including bone marrow chimerism and clodronate liposomes. Bone marrow chimerism capitalizes on the rapid turnover of PVM compared to microglia, resulting in a selective replacement of PVM with donor bone marrow derived cells (Hickey and Kimura, 1988; De Groot et al., 1992; Krall et al., 1994; Priller et al., 2001). Clodronate liposomes are injected intracerebroventricularly and are only phagocytosed by PVM resulting in their selective depletion via apoptosis (Polfliet et al., 2001a). Scavenger, chemotactic and antigen presentation functions of PVM have been probed in this way, as shall be discussed below.

	PVM	Microglia Resting/Active	Macrophages	Pericytes	Astrocytes	References
Macrophage:						
CD163	+++	hu ± rat –	hu ± rat –	–	–	(Graeber et al., 1989), Chapter 4
CR3	+	+/++	++	–	–	(Hohenester et al., 1999; Sasaki et al., 1996)
FcR	+	±	+	–	–	(Ulvestad et al., 1994a)
CD68	+++	±	+++	–	–	(Sasaki et al., 1996; Hulette et al., 1992; Graeber et al., 1989)
MR	+++	–	–	–	–	(Galea et al., 2005) (chapter 4)
APC:						
MHC I	++	±	+	–	–	(Gobin et al., 2001)
MHC II	++	±	++	–	–	(Gobin et al., 2001; Sasaki et al., 1996; Ulvestad et al., 1994b; Streit et al., 1989) (chapter 4)
DC SIGN	++*	–	+	–	–	(Schwartz et al., 2002) (chapter 4)
CD80	+*	–	+	–	±	(Zeinstra et al., 2003; De Simone et al., 1995) (chapter 4)
CD86	+*	–	+	–	– (+)°	(Zeinstra et al., 2003; De Simone et al., 1995) (chapter 4)
CD40	++*	–	+	–	– (+)°	(Gerritse et al., 1996) (chapter 4)
LCA	++	+	++	–	–	(Sasaki et al., 1996)
Microglia:						
Griffonia Symplicifolia	–	+++/+++	++	–	–	(Knabe and Kuhn, 1999; Streit, 1990)
RCA	–	+++/+++	++	±	±	(Hulette et al., 1992)
Pericytes:						
SMA	–	–	–	++	–	(Herman and D'Amore, 1985)
Astrocytes:						
GFAP	–	–	–	–	++	(Eng et al., 2000)

Tabel 1: *Markers to distinguish between PVM and other brain cells*
* A subpopulation of the perivascular macrophages is positive for this marker, ° Reactive astrocytes, Hu: human

2.1. Pathogen recognition

PVM express a multitude of receptors for pathogen-associated molecular patterns (PAMP) as well as for complement and IgG. They thus form an integral part of the innate immune defenses at the BBB. Such receptors enable the recognition and uptake of blood-borne or invading foreign particles as a result of ligation of pathogen receptors or opsonizing complement and IgG.

Both human and rodent PVM express the scavenger receptor (SR) A types I and II (Mato et al., 1996; Tomokiyo et al., 2002). These receptors are known to bind Lipid A, lipoteichoic acid (LTA) and CpG DNA, and thus internalize both Gram-positive and Gram-negative bacteria (Peiser et al., 2002). In SR A I/II knockout mice, PVM were noticed to have fewer lysosomal inclusions, indicating that these scavenger receptors are constitutionally active (Mato et al., 1997) (Chapter 4).

Our group recently showed that PVM in the human CNS express DC-SIGN (dendritic cell-specific ICAM (intracellular adhesion molecule)-3 grabbing nonintegrin (Chapter 4). DC-SIGN is a C-type lectin present on dendritic cells and involved in antigen capture as well as in T cell synapse formation (Geijtenbeek et al., 2000). DC-SIGN recognizes high mannose ligands on various pathogens, and may thus be responsible for scavenging pathogens that have penetrated the perivascular space in the CNS. Importantly in this respect, DC-SIGN acts as a receptor for HIV (Geijtenbeek et al., 2002; Chehimi et al., 2003) and it has been shown that perivascular cells in the brain are the main site of primary infection with this virus (Williams et al., 2001b).

Another receptor important for the recognition of pathogens that has been described in the murine CNS is the mannose receptor (MR) (Galea et al., 2005). The MR recognizes branched mannose containing carbohydrate structures on microbial antigens such as those from *Mycobacterium tuberculosis, Candida albicans, Pneumocystis carinii, Klebsiella pneumoniae* and *Streptococcus pneumoniae*, resulting in their receptor-mediated phagocytosis and enhanced microbicidal activity (Linehan et al., 2000). Interestingly most of these pathogens are known to infect the CNS. Indeed, PVM depletion using clodronate liposomes in a *Streptococcus pneumoniae* model in the rat resulted in a worsening of meningitis (Polfliet et al., 2001b).

CD14 is expressed by PVM in humans (Williams et al., 2001b) and most probably constitutively expressed by PVM in the rat (Lacroix et al., 1998), although the latter study used morphology and OX-42 immunoreactivity (i.e. complement receptor (CR) 3 positivity) to identify PVM. CD14 is known to bind lipopolysaccharide (LPS) complexed with LPS-binding protein. It is a glycosylphosphatidylinositol-anchored cell surface protein with no intracellular signalling domain but serves to concentrate and present LPS to toll-like receptor (TLR)-4. There are very few studies looking at the detailed *in situ* cellular expression of TLR in the brain. In the rat, TLR-4 gene expression was seen to occur constitutively in circumventricular organs and some parenchymal structures, but no colocalization was possible due to low levels of transcript (Laflamme and Rivest, 2001). Although TLR-4 expression should theoretically accompany CD14, this does not seem to be the case, since some CD14 positive areas were TLR-negative especially after endotoxin challenge (Laflamme and Rivest, 2001). On the other hand, TLR-4 was not immunocytochemically detectable in healthy human brain, but was evident in all active

Multiple Sclerosis (MS) lesions, especially in perivascular areas (Bsibsi et al., 2002): again no proper colocalization was done. In this study the same applied for TLR-3, which recognizes double stranded RNA. TLR-2, which is the PAMP receptor for lipoteichoic acid and peptidoglycan from Gram-positive bacteria, is expressed in the brain but has not been properly colocalized on a cellular basis (Laflamme et al., 2001).

Furthermore, rodent and human PVM express CD18/CD11b (Mac-1, CR3) and CD18/CD11c of the β2 integrin family, which act as receptors for complement fragments (iC3b), CD54 (ICAM-1), fibrinogen, bacterial LPS and altered proteins (Sasaki et al., 1996). Human PVM also display FcR enabling them to recognize IgG coated targets (Ulvestad et al., 1994a).

2.2. Antigen presentation

The presentation of antigens by PVM is of great importance since the normal healthy brain parenchyma lacks other antigen-presenting cells, which can initiate, amplify or regulate immune responses. Microglial cells in the CNS parenchyma are radically down-regulated cells and hardly express MHC molecules in normal conditions (Hickey and Kimura, 1988; Streit et al., 1989). Small numbers of dendritic cells in the healthy CNS are only found in the meninges and choroid plexus (Matyszak and Perry, 1996; McMenamin, 1999).

The location of the PVM at the BBB, together with its expression of MHC class II in several species (Streit et al., 1989; Hickey et al., 1992; Graeber et al., 1992; Ulvestad et al., 1994b; Sasaki et al., 1996) and of several costimulatory molecules, like CD40 and B7-2 (at least in the human, Chapter 4) (De Simone et al., 1995), supports an antigen-presenting role. Moreover, PVM have been seen to contact T cells in both rat (Walther et al., 2001) and human (personal observation) brain. Overall, this indicates that PVM might play an important role in the recognition of pathogens and degradation products transported through the bloodstream and hence form the first line of defense of the brain once the endothelial integrity of the barrier is damaged or circumvented (Angelov et al., 1998a).

There is functional evidence that PVM present brain-derived antigen to T cells that have been activated peripherally. Experimental autoimmune encephalomyelitis (EAE) developed normally in rat radiation bone marrow chimeras where the only potential antigen-presenting cells in the brain expressing the appropriate MHC molecules, restricting the infused encephalitogenic T cells, were the PVM (Ford et al., 1995). The disease in chimeras was clinically and neuropathologically identical to that in non-chimeric controls (Lassmann et al., 1993). Further confirmation for a functional role in antigen presentation by the PVM was obtained *in vitro* when rat brain macrophages were sorted into $CD45^{high}$ (bone-marrow derived macrophages including PVM) and $CD45^{low}$ (microglia) populations using flow cytometry: the $CD45^{high}$ population was the only one capable of stimulating myelin basic protein (MBP)-specific T cells to proliferate and secrete interleukin (IL)-2 (Ford et al., 1995).

2.3. Antigen transport

As described above PVM are professional scavengers of the perivascular space in the CNS. It is still unknown whether PVM transport antigens to draining lymphoid organs. In peripheral tissues the transport of antigens to draining lymphoid organs is performed by dendritic cells, which acquire their antigen in the target tissue. They then leave the tissue, especially if inflammation is present, and travel through the afferent lymph, where they occur as veiled cells, to reach the draining lymphoid organs.
The activation of naive T lymphocytes in the lymph nodes may result in an antigen specific immune response or in an antigen specific tolerance induction (Banchereau and Steinman, 1998; Steinman et al., 2000). It has been proposed that this mechanism also applies to brain-derived antigens, and that these antigens drain into the cervical lymph nodes (CLN) and are presented to recirculating lymphocytes, inducing T cells to target the brain (Phillips et al., 1997).

When CLN were removed from rats with EAE, this reduced the load of cerebral disease by 40%, probably because T cell priming was lacking (Phillips et al., 1997). Also, injecting a soluble antigen, albumin, in the grey matter of the brain of rats resulted in antibody formation in the CLN (Harling-Berg et al., 1989). These studies all suggest that the CLN play a key role in lymphocyte-mediated immune reactions in the brain. A study performed in the CLN of monkeys with EAE and in postmortem CLN of MS patients showed the presence of myelin antigens in cells expressing dendritic cell and macrophage markers as well as costimulatory molecules in the CLN (De Vos et al., 2002). Brain-derived antigens may potentially drain into the cervical lymph vessels either in cell-bound/intracellular form or else extracellular by bulk flow to be taken up by dendritic cells or macrophages inside the CLN.

Possible candidates for the cellular transport of antigens out of the brain are infiltrating monocytes or dendritic cells, microglia, and dendritic cells from the meninges or choroid plexus (Katz-Levy et al., 1999; Aloisi et al., 2000), as well as, PVM. The latter might travel via the perivascular space towards draining lymph vessels and then to the draining lymph node. However, this is merely speculative.

Starting from descriptive studies, which might provide clues for an antigen transport role of PVM, is MR expression. Ligand binding sites for the cysteine-rich domain of the MR have been detected in developing germinal centres in spleen, and have been followed on dendritic-like cells migrating from the subcapsular area of lymph nodes into follicular areas (Martinez-Pomares et al., 1996). It has thus been suggested that the MR directs antigen (bound by the carbohydrate recognition domains) towards sites of developing clonal immune responses (by the cysteine-rich domain). A fully functional soluble form of MR is generated by shedding of cellular MR by matrix metalloprotease activity (Martinez-Pomares et al., 1998) and a similar antigen transport potential has been suggested for soluble MR. Such a scenario is possible during CNS inflammation since interstitial fluid from the perivascular space is known to drain directly into nasal lymphatics and thus CLN through channels in the cribriform plate (Weller et al., 1996).

Functional studies addressing the issue of whether PVM mediate antigen drainage are lacking. Kida *et al.* have observed PVM in healthy rats labelled with intrastriatally injected

Indian ink to persist for years (Kida et al., 1993), but this does not exclude the possibility that some PVM exit the brain. On the other hand, pathology seems to lower the threshold for the exit of potentially antigen-presenting cells from the brain. Thus, donor MHC I positive cells were observed in the lymph nodes and spleen of rats which received an allogeneic CNS graft in the forebrain and underwent rejection (Broadwell et al., 1994). In the periphery, it is known that pathogen products such as LPS or cytokines such as IL-1 or TNF-α induce dendritic cell migration into the T cell area of lymphoid organs (Cumberbatch and Kimber, 1995; Banchereau et al., 2000).

2.4. Maintenance of the BBB

Macrophages in peripheral tissues are a heterogeneous cell population and can be activated via various routes, including classical activation routes induced by e.g. lipopolysaccharide (LPS) or interferon (IFN)γ and alternative activation routes induced by e.g. IL-4 or IL-13 (Gordon, 2003). MR and CD163 expression in macrophages denote a state of "alternative activation" (Gordon, 2003). This state is characterized among other things by marked upregulation of arginase-1 that results in proline production and gives the alternatively activated type 2 macrophages a pro-fibrotic tendency (Hesse et al., 2001). In normal physiology this production of extracellular matrix might contribute to maintenance of the BBB since the PVM is sandwiched between glial and endothelial basement membranes. Indeed, SR A knockout mice which have an abnormal number and phenotype of PVM are characterized by a thin, absent or discontinuous glial basement membrane (Mato et al., 1997).

2.5. Immune-to-brain signalling

Peripheral infection or inflammation results in a constellation of symptoms known as sickness behaviour, which includes fever, lethargy, anhedonia, hyperalgesia, reduced physical activity and social withdrawal. This is a normal physiological response and does not represent a diseased state of the CNS. Given the presence of the BBB, mechanisms must be in place to enable the brain to sense circulating PAMP and cytokines. Numerous studies have shown that these mediators first activate cells lying at key areas of the brain-immune interface (circumventricular organs, meninges and BBB) initiating a wave of inflammation, which then penetrates the brain parenchyma proper, thus constituting a biphasic response. The first phase usually occurs at around 1-2 hours following the peripheral immune stimulus and it then dies down to be followed by a second phase a few hours later. This has been shown to be the case using different markers including c-fos mRNA (Herkenham et al., 1998), I-κB mRNA (Quan et al., 1997; Laflamme and Rivest, 1999), IL-1β mRNA (Quan et al., 1998), IL-1β protein (Konsman et al., 1999), tumour necrosis factor (TNF)-α mRNA (Breder et al., 1994; Nadeau and Rivest, 1999) and CD14 mRNA (Lacroix et al., 1998).

PVM are in a state of readiness to respond to inflammatory stimuli. They express the whole plethora of PAMP receptors discussed above as well as the type 1 IL-1 receptor (Schiltz and Sawchenko, 2002). Once stimulated, they can secrete IL-1β (van Dam et al., 1992; Bauer et al., 1993; Angelov et al., 1998b) and TNF-α (Mato et al., 1998) and express immunocytochemically detectable amounts of cyclo-oxygenase-2 (COX-2)

(Elmquist et al., 1997; Schiltz and Sawchenko, 2002) and inducible nitric oxide synthase (Mato et al., 1998). Cerebral endothelial cells, while capable of responding to inflammatory stimuli (Matsumura et al., 1998; Ek et al., 2001), are not as immunologically alert as PVM, which are the most constitutively immunophenotypically activated cells in the CNS (Perry, 1998). PVM thus have the potential for amplification of weak peripheral cytokine signals at the BBB. Using double immunocytochemistry, it has been shown that PVM upregulate COX-2 as soon as 30 minutes after intravenous IL-1β injection in the rat, reaching a peak at 2 hours, and persisting as long as 4-6 hours post-injection (Elmquist et al., 1997; Schiltz and Sawchenko, 2002). It thus seems that PVM activation bridges both phases of the innate immune response at the BBB and suggests an amplification role connecting the two phases.

3. PVM in disease

We feel it is important to distinguish between PVM in health and in disease. Mention has already been made above of situations in which phenotype and function of PVM differ depending on the presence or absence of inflammation, for example TLR-3 and TLR-4 expression. Moreover, the vast majority of surface molecules, which have been discussed are upregulated in disease (Williams et al., 2001a).

3.1. Bacterial meningitis

When perivascular and meningeal macrophages were selectively depleted in a rat model of pneumococcal meningitis this resulted in deterioration of clinical symptoms, due to a decreased recruitment of polymorphonuclear cells to the meninges and decreased elimination of pneumococci (Polfliet et al., 2001b). This study indicates that PVM are involved in the recruitment of granulocytes during meningitis, though this was not due to decreased production of the predominant neutrophil chemokine MIP-2 (macrophage inflammatory protein-2). Disease worsening could also have occurred as a result of ineffective MR-dependent bacterial clearance (see above). Nevertheless, PVM are known to secrete chemokines such as MIP-1α and RANTES (regulated upon activation, normal T cell expressed and secreted) under pathological conditions (Boven et al., 2000; Walther et al., 2001). Functional studies of PVM's chemotactic potential are however lacking.

3.2. Autoimmune disease

EAE is an autoimmune inflammatory disorder of the CNS, commonly used as a model of MS. When acute EAE is induced in the Lewis rat by immunization with MBP in complete Freund's adjuvant, this results in perivascular inflammation in the CNS, characterized by mononuclear cell infiltrates consisting of T lymphocytes and macrophages. The animals develop transient neurological deficits around day 10 after immunization, which disappear 5 days later (Polfliet et al., 2002). However, a robust increase in ED2 expression following immunization occurs before any cellular infiltration or clinical signs of EAE (Polfliet et al., 2002). Interestingly, it has been shown that EAE in mice is similarly preceded by a behavioural syndrome (Pollak et al., 2000), which is in support of an immune-to-brain signalling role for PVM.

Experiments in our lab showed that after the selective depletion of PVM and meningeal macrophages in EAE, the initial phase of the disease was suppressed (Polfliet et al., 2002). This might suggest an important *early* chemotactic role of PVM; later, once the presence of activated T cells in the perivascular space is established, this results in self-perpetuating chemokine secretion (Karpus and Ransohoff, 1998), which over-rides any contribution from PVM. However, it is clear from this functional study that the PVM's chemotactic role is largely redundant. Another mechanism by which macrophages contribute to cellular infiltration maybe their early expression of IL-1 during EAE, which is known to upregulate ICAM-1 on the endothelial cells of the BBB (Bauer et al., 1993).

Similar to our findings in EAE, we observed an increased number of CD163-positive macrophages in MS brains (Chapter 4). Moreover, PVM upregulated their expression of the costimulatory molecules CD40, CD80, CD86 and the antigen recognition molecule DC-SIGN (Chapter 4). Both *in vivo* animal and post mortem human studies therefore point towards a significant role of PVM in CNS autoimmunity.

3.3. Retroviral encephalitis

HIV encephalitis is clinically evident as HIV-associated dementia. Given the presence of the BBB, the entry of HIV into the CNS must involve an alternative route. In SIV encephalitis, it has been shown that PVM are the primary cell type that is productively infected (Williams et al., 2001b). Indeed, PVM express a variety of surface molecules, which are used as receptors by HIV/SIV to gain entry into the cell. These include CD4, several chemokine receptors (CCR3, CCR5, CXCR4) (Westmoreland et al., 1998) and DC-SIGN (Geijtenbeek et al., 2002; Chehimi et al., 2003). Moreover, there is recent circumstantial evidence that the physiological replenishment of PVM by circulating monocytes, which is augmented in inflammation, might be a mechanism behind primary CNS infection by the virus (Kim et al., 2003). Thus HIV-infected bone marrow derived monocytes act as "Trojan horses" that traffic to the CNS to become PVM.

3.4. Lysosomal storage disorders

The G_{M2} gangliosidoses, such as Tay-Sachs disease, are lysosomal storage disorders characterized by the intracellular accumulation of the ganglioside G_{M2} and related glycosphingolipids in the CNS (Jeyakumar et al., 2002). Clinical features include epilepsy, motor/cerebellar abnormalities and cognitive dysfunction. These diseases arise from inherited deficiencies in lysosomal β-hexosaminidase, which results in the accumulation of neuronal inclusions leading to neurodegeneration. In a mouse model of Tay-Sachs disease, PVM have been shown to contain microcorpuscular material morphologically identical to that seen in neurons (Mato et al., 2002). This suggests that PVM might be directly involved in processing secreted material or dying neurons.

4. Conclusion

There is reasonable circumstantial evidence that PVM represent an important immunological player at the BBB. However, solid experimental evidence proving the PVM's role in perivascular inflammation in the CNS is limited. The recent identification of specific markers for this cell population and the development of methods to study their functional role in *in vivo* animal models now open new perspectives for research on this interesting cell population (Hulette et al., 1992).

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3.

The macrophage scavenger receptor CD163

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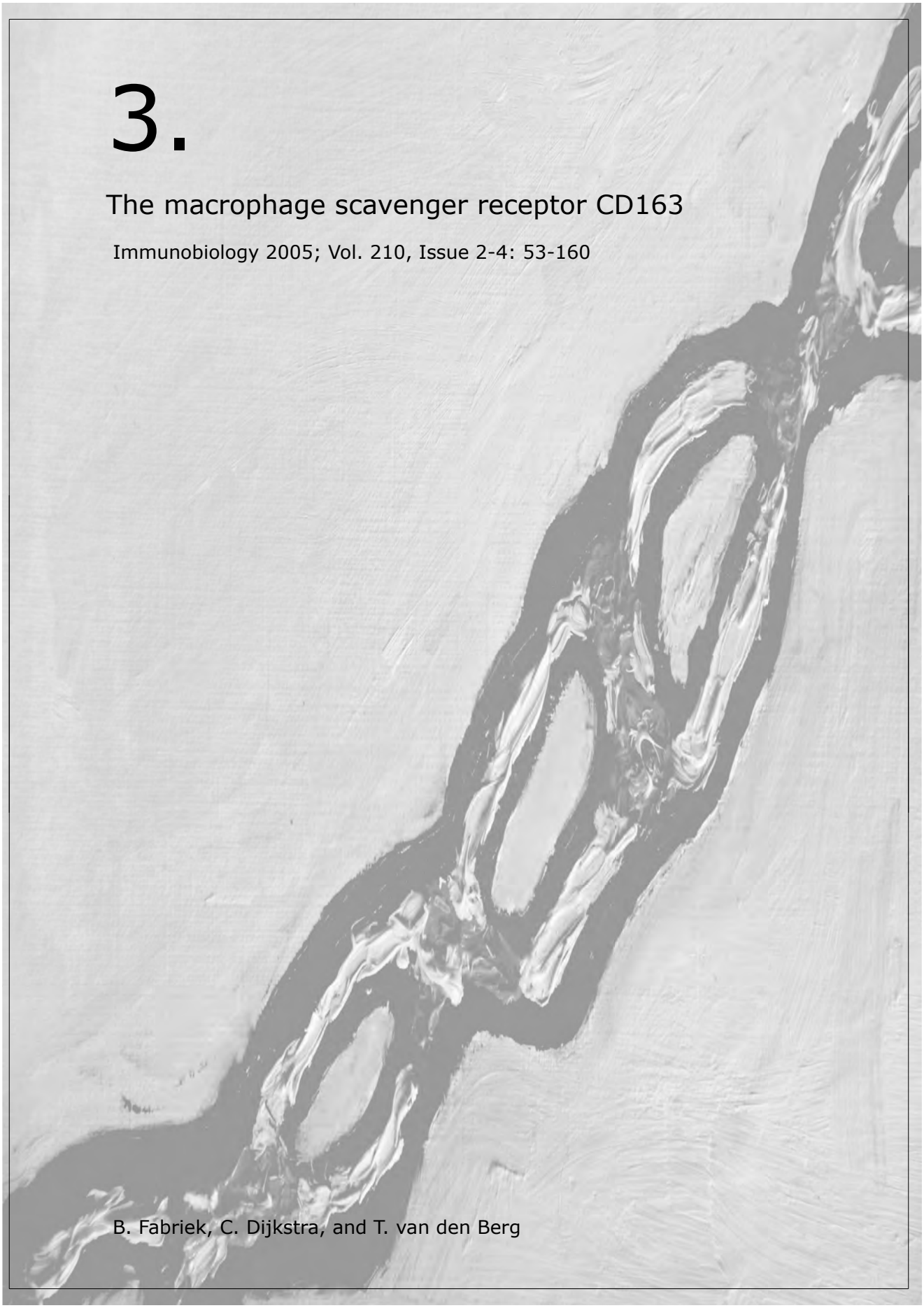

B. Fabriek, C. Dijkstra, and T. van den Berg

Abstract

Mature tissue macrophages form a first line of defense to recognize and eliminate potential pathogens; these specialized cells are capable of phagocytosis, degradation of self and foreign materials, establishment of cell-cell interactions, and the production of inflammatory mediators. Mature tissue macrophages express a variety of receptors, including the scavenger receptor cysteine-rich (SRCR) superfamily members. CD163 is a member of the SRCR family class B and is expressed on most subpopulations of mature tissue macrophages.

The best characterized function of CD163, which is essentially a homeostatic one, is related to the binding of Hemoglobin: Haptoglobin complexes. Furthermore, it has been suggested that CD163-positive macrophages play a role in the resolution of inflammation, as they are found in high numbers in inflamed tissue.

1. Mature tissue macrophages

Macrophages play an important role in normal homeostasis and during various pathological conditions such as infection, (chronic) inflammation, atherosclerosis and cancer. While macrophages originate from a common myeloid progenitor cell in the bone marrow, mature macrophages constitute a very heterogeneous population of cells with respect to morphology, phenotype and function. Monocytes and the macrophage subsets of the serous cavities (alveolar, pleural and peritoneal macrophages) have been relatively well studied, largely because they can be (relatively) easily obtained. In contrary, very little is known about the different macrophage subpopulations that occupy the various parenchymal organs.

Although these mature tissue macrophages, as we will collectively call them, may simply form a first line of defense to recognize and eliminate potential pathogens, many of these are also believed to serve specialized homeostatic functions. Mature tissue macrophages are capable of phagocytosis, degradation of self and foreign materials, establishment of cell-cell interactions, and the production of inflammatory mediators (Gordon et al., 1995). For instance, splenic red pulp macrophages are believed to play a major role in the clearance of aged erythrocytes, thymic cortical macrophages remove apoptotic thymocytes that have failed to survive positive and negative selection, osteoclasts play a critical role in bone formation and remodelling, and resident bone marrow macrophages mediate interactions with erythroblasts that appear to be essential for erythroid development.

The specialized functions of macrophage subsets are reflected in the molecular "tools" (receptors, signalling components, etcetera) they express, such as Fc-, complement-, and scavenger receptors, adhesion molecules, and receptors for soluble mediators such as cytokines, chemokines, prostaglandins, and growth factors. The expression of the different receptors varies depending on the tissue localization and activation status of the macrophage (Gordon et al., 1995). The characterization of such molecules provides clues about the functional specialization of the macrophages that express them. Here, we will focus on one of these, CD163, a scavenger receptor family member that is expressed on most subsets of myeloid cells.

2. Scavenger receptor cysteine-rich superfamily

The initial interaction of macrophages with a newly invaded pathogen is mediated by pattern recognition receptors. Scavenger receptors belong to the group of pattern recognition receptors and are known for their broad range of ligand binding. In the group of scavenger receptors a subgroup of scavenger receptor cysteine-rich (SRCR) receptors can be identified. The SRCR superfamily is a family of structurally related transmembrane glycoproteins. The characteristic building block of the extracellular domain of these molecules is the SRCR domain, which is an ancient and highly conserved domain of approximately 110 residues (Krieger, 1997). These SRCR domains form a common structural fold, as has been revealed for instance from the crystal structure of Mac-2 binding protein, which can be described as a six-stranded β sheet cradling an α-helix. The loop connecting α strand 5 and 6 has been suggested as a candidate site for molecular interaction (Hohenester et al., 1999). Many SRCR family

members serve a role in innate host defense by acting as pattern recognition molecules, sometimes with a relatively broad specificity. Others mediate selective protein-protein interactions (Krieger, 1997).

There are two variants of the SRCR domain that divide the SRCR molecules into two groups; group A and group B. Both group A and group B have 3 disulfide bridges in common, but the group B SRCR molecules contains a fourth disulfide bridge, and thus the two groups can be distinguished having 6 and 8 cystein residues, respectively (Resnick et al., 1994; Aruffo et al., 1997). The group A SRCR molecules include SR-AI, Mac-2 binding protein, MARCO, Speract receptor (only in sea urchin), lysyl oxidase related protein, complement factor I, and enterokinase. With respect to ligand specificity several members of the group A SRCR domain receptors, like MARCO and SR-AI, which are both expressed by typical subsets of mature tissue macrophages, are particularly promiscuous and ligands include proteins, polyribonucleotides, polysaccharides, and lipids for which the main common feature is that they are 'polyanionic' (Resnick et al., 1994; Krieger, 1997; Elomaa et al., 1998; Sankala et al., 2002). Ligand binding may involve the SRCR domains, as has been demonstrated for MARCO, but unexpectedly in case of SR-AI it is SRCR domain-independent and mediated by the collagen domain of the molecule. Upon ligand binding MARCO and SR-AI/II generally mediate uptake and lysosomal degradation.

The group B SRCR receptors include CD5, CD6, SPα, WC1 (bovine), gall-bladder mucin (bovine), Perma-SPERG (only in sea lamprey), Ebnerin (rat), CRP-ductin (murine), Hensin (rabbit), gp-340, M160, and CD163 (see figure 1) (Jones et al., 1986; Wijngaard et al., 1992; Mayer and Tichy, 1995; Li and Snyder, 1995; Cheng et al., 1996; Takito et al., 1996; Aruffo et al., 1997; Gebe et al., 1997; Mollenhauer et al., 1997; Hogger et al., 1998a; Holmskov et al., 1999; Gronlund et al., 2000). Within this class B scavenger receptor family CD163 and M160 are the only members selectively expressed on monocytes and macrophages (Van den Heuvel et al., 1999; Gronlund et al., 2000). The other members are expressed on other immune cells or on non-hematopoietic cells, e.g. CD5 and CD6 are predominately expressed on B and T lymphocytes, WC1 is expressed on CD4-CD8 $\gamma\delta$ T lymphocytes (Resnick et al., 1994) and the secreted gp-340 is expressed by mucosal epithelial cells. Little is known about the functions of members of the group B SRCR family. Ligands that have thus far been described include the endothelial adhesion receptor ALCAM (CD166) for CD6 (Aruffo et al., 1997), for gp-340 IgA and bacteria have been identified (Bikker et al., 2002), for Spα and a unidentified ligand on monocytes is present (Gebe et al., 1997), and several ligands for CD5 (e.g. Immunoglobin heavy-chain variable framework regions, CD72 and a unknown ligand on T and B cells) (Raman, 2002). In general, it seems clear that the SRCR domains have evolved to recognize a large variety of structurally different ligands, with some being quite promiscuous and others having a relatively narrow specificity. One major challenge will be to explore the full potential of the SRCR family in terms of ligand recognition and to understand the physiological relevance of these interactions.

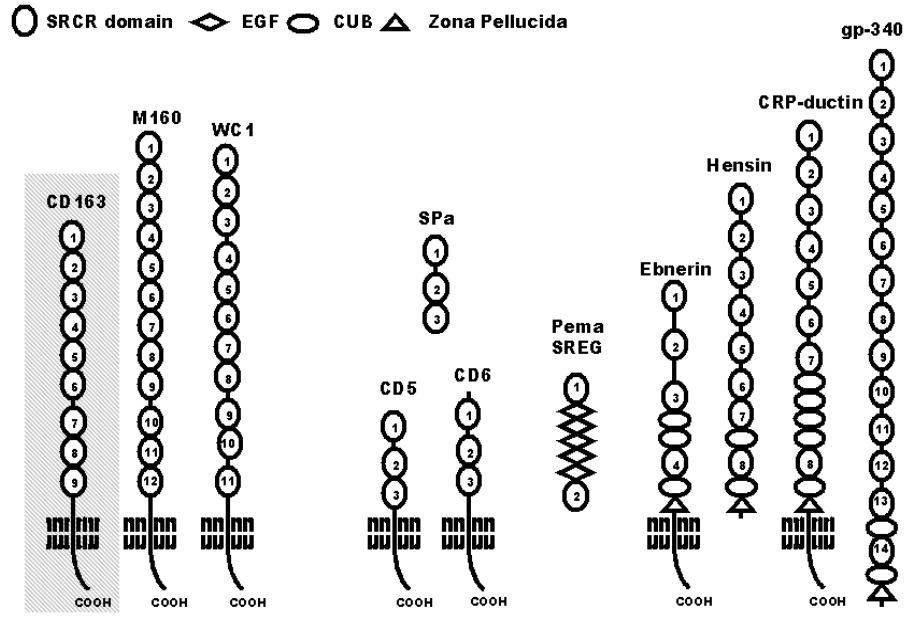

Figure 1: *Schematic representation of the members of the Group B SRCR superfamily*
Based on a review by Aruffo and co-workers (Aruffo et al., 1997). Abbreviations: EGF: Epidermal Growth Factor, CUB: complement component Cr1/C1s, sea urchin protein, and bone morphogenic protein.

3. CD163: structure, expression and regulation

As described above, CD163 (also known as RM3/1, hemoglobin scavenger receptor (HbSR), M130, or p155) is a member of the SRCR family class B expressed on subpopulations of mature tissue macrophages. To date, CD163 has been described in human and marmosets, but also porcine and rodent homologues have been reported (Zwadlo-Klarwasser et al., 1992; Schaer et al., 2001; Sanchez-Torres et al., 2003).

In rat, CD163 has been described as ED2 antigen (Chapter 9) The monoclonal antibody ED2 is used to define mature tissue macrophages in the rat since ED2, in contrary to humans, is not present on monocytes and alveolar macrophages (Van den Heuvel et al., 1999; Buechler et al., 2000). In humans, CD163 is a 130-kDa glycoprotein containing a single transmembrane element, a short cytoplasmic tail and a large extracellular region of 9 SRCR domains (Law et al., 1993; Hogger et al., 1998a; Hogger et al., 1998b). 4 splice variants have been identified that differ in the length of their cytoplasmic tail (Ritter et al., 1999).

The expression of CD163 has been most extensively studied in man (Van den Heuvel et al., 1999) and in rat (Dijkstra et al., 1985) (Chapter 8). As mentioned above, the expression of CD163 is restricted to cells of the monocytic lineage. Mature tissue macrophages that express high levels of CD163 include, for instance, Kupffer cells in the liver, red pulp macrophages in the spleen, cortical macrophages of the thymus, resident mature bone marrow macrophages, perivascular and meningeal macrophages

of the central nervous tissue (not microglia), and scattered macrophages in various other tissues (Van den Heuvel et al., 1999) (Table 1). CD163-positive macrophages are also found during the healing phase of acute inflammation, in chronic inflammation and in wound healing tissue, whereas freshly infiltrated macrophages are CD163-negative (Verschure et al., 1989). It has been suggested that CD163-positive macrophages play a role in the resolution of inflammation, as they are found in high numbers in inflamed tissue. In addition, during the resolution phase of the systemic inflammatory response to cardiopulmonary bypass surgery and coronary artery bypass graft surgery elevated levels of CD163 on monocytes were found (Goldstein et al., 2003; Philippidis et al., 2004). On the other hand, the upregulation of CD163 on perivascular macrophages during Multiple Sclerosis (Chapter 4) and experimental autoimmune encephalomyelitis in rat is one of the first locally detectable events in the inflammatory process (Polfliet et al., 2002).

Tissue	**Macrophage subpopulation**	**CD163**
Spleen	Red pulp macrophages	+
	Perifollicular macrophages [1]	-
	Marginal zone macrophages [2]	-
	Marginal metallophilic macrophages [2]	-
Lymph nodes	Medullary macrophages	+
	Perifollicular macrophages	+
Thymus	Medullary macrophages	+
	Cortical macrophages	+
Liver	Kupffer cells	+
Brain	Perivascular macrophages	+
	Meningeal macrophages	+
	Microglia	-
Lung	Alveolar macrophages [1]	+
	Interstitial macrophages	+
Peritoneum	Peritoneal macrophages [2]	+ (15-40%)
Blood	Monocytes [1]	+ (10-30%)

Table 1: *Distribution of CD163-positive macrophages in the most common studied tissues in rat and human.* [1] Only present in human. [2] Only present in rat.

The expression of CD163 can be regulated by a variety of factors, which has been studied extensively *in vitro*. Consistent with the expression on mature macrophages *in vivo*, the *in vitro* differentiation of monocytes to macrophages strongly induces CD163 mRNA and protein expression. After *in vitro* treatment of monocytes with glucocorticoids the percentage of CD163 positive monocytes has been reported to rise from 10-30% to 90% (Wenzel et al., 1996). When glucocorticoids are injected *in vivo* into human volunteers this also results in an increase of CD163-positive monocyte population of more than 80% within 6 hours (Zwadlo-Klarwasser et al., 1990) (Chapter 7). Interestingly, other anti-inflammatory mediators such as IL-10 and IL-6 also promote CD163 expression (Buechler et al., 2000; Sulahian et al., 2000). Inversely, pro-inflammatory mediators, like LPS, IFN-α, and TNF-γ, suppress CD163 mRNA and protein (Buechler et al., 2000).

When monocytes are differentiated towards dendritic cells (by GM-CSF and IL-4) CD163 mRNA and protein levels are also suppressed (Buechler et al., 2000), although

a report by Sulahian and co-workers indicate that monocyte-derived dendritic cells may still express low levels of CD163 (Sulahian et al., 2000). When monocytes are stimulated to a 'classical' or 'alternative' phenotype, by respectively IFN-γ and LPS or IL-4 and IL-13, a particularly high expression of CD163 is seen in "alternatively activated'' macrophages, which has been suggested to play a major role in dampening the inflammatory response and in the scavenging components of damaged cells (Gordon, 2003). The regulation of CD163 by pro- and anti- inflammatory mediators suggests a link between CD163 immune suppression and the resolution of inflammation. The fact that CD163 is expressed at high levels on most subsets of mature tissue macrophages would rather point to a role in recognition of pathogens and subsequent innate immune response.

3.1. The function of CD163 in host defense and homeostasis

Although there is some evidence that porcine CD163 may function as a receptor for the African swine fever virus (Sanchez-Torres et al., 2003), there is no evidence for a more generalized and direct role in infection. It has however, been shown that triggering can regulate macrophage cytokine production, but a direct contribution to host defense or inflammation has not been established (Van den Heuvel et al., 1999; Madsen et al., 2004).

Thus far, the best characterized function of CD163, which is essentially a homeostatic one, is related to the binding of Hemoglobin: Haptoglobin (Hb-Hp) complexes. Senescent or malformed red blood cells are cleared by tissue macrophages in the spleen, liver and bone marrow. Intravascular hemolysis generates the oxidative and toxic free Hb, which can be complexed to Hp for clearance by tissue macrophages (Gordon, 2001). CD163 has been identified as a receptor involved in clearance and endocytosis of these Hb-Hp complexes by resident macrophages, and may thereby protect tissues from free Hb-mediated oxidative damage (Madsen et al., 2001; Kristiansen et al., 2001; Graversen et al., 2002). The Hb-Hp binding to CD163 is of high affinity (Kd (Hb-Hp 2:2); 0.2 nM), requires the presence of calcium (Kristiansen et al., 2001), and can be effectively inhibited by antibodies binding to SRCR domain 3. Proteolytic cleavage in domain 3 inactivates ligand binding, so it can be concluded that SRCR domain 3 is a critical determinant for the calcium dependent recognition of Hb-Hp complexes (Madsen et al., 2004).

Besides having a protective detoxification effect by removing Hb from the plasma, the CD163-mediated endocytosis of Hb-Hp may also represent an important pathway for the uptake of iron in tissue macrophages, which in turn maybe important for the recycling of iron. It is further of interest that humans have two polymorphic Hp variants designated Hp1 and Hp2, CD163 shows a greater affinity for Hb-Hp (2-2) complexes than for Hb-Hp (1-1) complexes (Kristiansen et al., 2001). Furthermore it is of interest that carriers of the Hp2-2 genotype have an increased risk for diabetic cardiovascular disease (Asleh et al., 2003), it remains however unclear whether and how this is related to CD163.

After the endocytosis of Hb-Hp, the heme subunit of hemoglobin is degraded by heme-oxygenase (HO) enzymes. Two isoforms of HO have been identified, HO-2 which

is constitutively present under normal physiological conditions and HO-1 which is inducible by anti-inflammatory stimuli, such as glucocorticoids and IL-10, often present in macrophages during inflammation (Wagener et al., 2003; Philippidis et al., 2004). The breakdown of heme yields biliverdin, free iron, and carbon monoxide, which have anti-inflammatory effects. Interestingly ligation of CD163 by Hb-Hp complexes elicits a direct production of cytokines (Madsen et al., 2004), including IL-10, which in turn may further enhance CD163 and HO expression. It seems possible that this creates a positive feedback loop that may function to overcome oxidative damage by free Hb or perhaps other toxic agents during inflammation as well (Philippidis et al., 2004). It should be noted that at present still little is known about the signalling pathways regulated by CD163 to induce cytokine production. Thus far, it is known that cross-linking of CD163 by monoclonal antibodies induces a transmembrane signal, casein kinase II and protein kinase C dependent intracellular calcium mobilization and IP3 synthesis, resulting in macrophage activation and secretion of pro-inflammatory cytokines, such as IL-6, IL-1β and GM-CSF (Van den Heuvel et al., 1999; Ritter et al., 2001). Interestingly, cross-linking of antibodies to CD163 results in different cytokine profiles than cross linking with Hb-Hp, it is unknown whether this is caused by a direct receptor-mediated signal or an indirect heme-mediated cellular response.

Another function of CD163, described by Hogger and co-workers is that the RM3/1 antibody, recognizing CD163 (Hogger et al., 1998a), was able to inhibit the adhesion of dexamethasone treated monocytes to activated endothelial cells (Hauptmann et al., 1994; Wenzel et al., 1996), suggesting that CD163 may perhaps have cellular ligands and somehow play a role in transendothelial migration. This issue clearly awaits confirmation and a more extensive analysis.

4. Soluble CD163

CD163 is like most members of the SRCR family, expressed as a membrane bound protein, but until now is the only scavenger receptor that has been reported to be actively shed from the cell surface. The treatment of CD163-positive monocytes with phorbol 12-myristate 13-acetate (PMA) or the stimulation of FcγR stimulation results in a rapid release of surface CD163 (Droste et al., 1999; Sulahian et al., 2000; Sulahian et al., 2004). This shedding is protein kinase C dependent and can be blocked by protease inhibitors (Droste et al., 1999). Furthermore, tissue inhibitors of metalloproteinases (especially TIMP-3) prevented shedding of CD163 from monocytes and macrophages, which is therefore thought to be catalyzed by membrane-bound metalloproteinases, ADAMs (Matsushita et al., 2002). This suggests a role for metalloproteinases in the shedding of CD163 from monocytes and macrophages.

It seems likely that this proteolytic shedding accounts for the soluble CD163 (sCD163) that is found in plasma at relatively high concentrations (median 1.9 mg/l), even under normal conditions (Droste et al., 1999; Kristiansen et al., 2001; Moller et al., 2002b). In a number of pathological conditions the levels of sCD163 are significantly increased. Firstly, elevated concentrations are found in hematological patients (Droste et al., 1999; Kristiansen et al., 2001; Moller et al., 2002b). sCD163 is also elevated in patients with sepsis, myeloid leukaemia, and in patients with Gaucher

disease (Moller et al., 2004) which are all diseases with proliferation of cells of myelo-monocytic origin in which enhanced sCD163 could perhaps be explained by elevated numbers of monocytes (Moller et al., 2002a). Furthermore, the levels of sCD163 are higher in sera of rheumatoid arthritis (RA) patients and spondylarthropathy synovitis patients than sera of healthy controls (HC). Of interest, levels of sCD163 in RA synovial fluids were consistently higher than in paired serum samples; supporting the hypothesis that additional sCD163 is predominantly shed by local inflammatory synovial lining macrophages (Matsushita et al., 2002; Baeten et al., 2004).

Overall sCD163 does not correlate with CRP, indicating that sCD163 does not directly reflect the acute phase response of inflammation (Moller et al., 2002a), whereas a study performed in RA patients did show an association with CRP. CRP in RA is known as a reliable marker for the disease activity (Matsushita et al., 2002). We have also obtained evidence for an elevation of sCD163 in Multiple Sclerosis (Chapter 6). Again, the observed enhancement of sCD163 is likely to be the results of shedding of CD163 at the inflamed site, in this case the central nervous system (chapter 4).

Apart from the above, there is also evidence for sCD163 regulation in acute inflammation. When volunteers are given LPS intravenously this causes as an increase in sCD163 levels (Hintz et al., 2002), in long-term cultures LPS gives an increase in sCD163 whereas the direct effect is a decrease in sCD163 (Sulahian et al., 2004). The inverse relations between membrane-bound CD163 expression and sCD163 expression in human blood suggested that sCD163, at least during this condition of acute inflammation, was derived from circulating monocytes and tissue macrophages (Davis and Zarev, 2005).

It should be emphasized that, in spite of the relatively high concentrations in plasma the functional relevance of sCD163 is presently unknown. Clearly, proteolytic cleavage may act as a feedback mechanism to reduce the level of functional CD163 on macrophages. Perhaps this would be important if CD163 were indeed contributing significantly to for instance cytokine production. Another contribution is that sCD163 may compete with surface CD163 for ligand binding (Frings et al., 2002). Finally, there is some evidence that sCD163 can negatively regulate phorbol ester-induced human T-lymphocyte activation *in vitro* (Hogger and Sorg, 2001), but this observation has not been confirmed by others.

5. Concluding remarks

Taken together, CD163 is a scavenger receptor class B member expressed selectively on most subpopulations of mature tissue macrophages. It functions as a receptor for Hb-Hp complexes and as such appears to participate in the clearance of free Hb, thus preventing oxidative tissue damage. In addition, CD163 may play a role in host defense, for instance by regulating the release of cytokines by macrophages. Further evidence for example from *in vivo* studies with CD163-mutant animals, should provide insight into the actual role of this molecule and the mature tissue macrophage subsets that express it.

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4.

CD163-positive-perivascular macrophages in the human CNS express molecules for antigen recognition and presentation

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Abstract

Perivascular macrophages (PVM) constitute a subpopulation of resident macrophages in the central nervous system (CNS) that by virtue of their strategic location at the blood-brain barrier potentially lend themselves to a variety of important functions in both health and disease. Functional evidence suggests that PVM play a supportive role during experimental autoimmune encephalomyelitis in rodents. However, the function of PVM in the human CNS remains poorly characterized.

We first set out to investigate the validity of the antibody EDHu-1, which recognizes human CD163, to specifically identify human PVM. Secondly, we wanted to gain insight into the function of PVM in antigen recognition and presentation and therefore we studied the expression of DC-SIGN, mannose receptor, MHC class II and several costimulatory molecules by PVM in the normal and inflamed human CNS (Multiple Sclerosis brain lesions). Conventional immunohistochemistry and double labelled immunofluorescence techniques were used.

We show that CD163 specifically reveals PVM in the normal human CNS. In MS lesions, CD163 staining reveals expression on foamy macrophages and microglia, besides an upregulation of the amount of PVM stained. On the other hand, mannose receptor expression is restricted to PVM in both normal and inflamed brain tissue. Furthermore, we show that a subpopulation of PVM in the human brain express several molecules involved in antigen recognition, presentation, and costimulation. Therefore PVM, which occupy a strategic location at the BBB, are equipped to recognize antigen and present it to T cells, supporting a role in the regulation of perivascular inflammation in the human CNS.

Introduction

The central nervous system (CNS) contains three populations of resident macrophages: microglial cells, meningeal macrophages and perivascular macrophages (PVM) (Bauer et al., 1995). PVM can easily be distinguished from microglia (Hickey and Kimura, 1988) and pericytes by their location, morphology and phenotype (Graeber et al., 1989). PVM are big, round or oval cells that lie between the endothelial and glial basement membranes of cerebral blood vessels. Pericytes are positioned between endothelial cells and the vascular basement membrane and contain smooth muscle elements (Kida et al., 1993; Mato et al., 1996). Microglial cells have slender branching processes and are dispersed throughout the brain parenchyma.

At the blood-brain barrier (BBB), PVM occupy a strategic position to encounter hematogenous pathogens and control innate and adaptive immune responses in the brain. Functional evidence that PVM can possibly present antigen in rats come from studies using clodronate liposomes. After selective depletion of perivascular and meningeal macrophages in experimental autoimmune encephalomyelitis (EAE) rats by clodronate liposomes (Polfliet et al., 2001), the clinical symptoms of EAE were delayed and suppressed (Polfliet et al., 2002). In the healthy CNS, dendritic cells which are commonly considered as the professional antigen-presenting cells (APC) of the immune system, can only be found in the meninges and choroid plexus (Matyszak and Perry, 1996; McMenamin, 1999) and in contrast to all other body tissues, brain parenchyma is devoid of typical dendritic cells (Aloisi et al., 2000). PVM are likely candidates fulfilling this antigen-presenting role. We were interested in studying such a potential role of PVM in the human CNS, and therefore set out to investigate the expression of molecules with known functions in antigen recognition and presentation on PVM.

Receptors important for the recognition of pathogens are the C-type lectins DC-SIGN (dendritic cell-specific ICAM (intracellular adhesion molecule)-3 grabbing nonintegrin) and the mannose receptor (MR). DC-SIGN is present on dendritic cells and is involved in antigen capture as well as in T cell synapse formation (Geijtenbeek et al., 2000). The MR is expressed by specific macrophage subpopulations (Linehan et al., 1999) and is involved in pathogen recognition and clearance, as well as adhesion (Linehan et al., 2000).

PVM express major histocompatibility complex (MHC) class II as demonstrated in several species (Streit et al., 1989; Hickey et al., 1992; Graeber et al., 1992; Ulvestad et al., 1994; Sasaki et al., 1996) and this supports an antigen-presenting role. Besides MHC class II, the expression of costimulatory molecules, like CD80 (B7-1) and CD86 (B7-2) are necessary for effective antigen presentation. In inflammatory diseases, like Multiple Sclerosis (MS), these molecules are upregulated on activated microglia and infiltrating macrophages (Windhagen et al., 1995; De Simone et al., 1995), but their expression by human PVM has not been studied in detail.

In order to study the properties of PVM in the human CNS in detail, it was important to establish an appropriate marker for PVM. Previous studies in the rat have shown that the monoclonal antibody (mAb) ED2 (Dijkstra et al., 1985; Barbe et al., 1990) selectively identifies rat PVM. Purification and sequencing of the ED2 antigen revealed

its identity as rat CD163 (Chapter 9). CD163 is a membrane glycoprotein belonging to the scavenger receptor cysteine-rich (SRCR) superfamily group B. It has been identified as a hemoglobin-haptoglobin scavenger receptor (Kristiansen et al., 2001) and is involved in a signal transduction pathway resulting in the production of cytokines (Ritter et al., 2001). We therefore investigated whether CD163 might be a selective marker for PVM in the human CNS using the mAb EDHu-1 (Van den Heuvel et al., 1999).

In this study we show that CD163 is specifically expressed by PVM in the normal human CNS. However, in inflamed tissue such as MS lesions, CD163 is also expressed by foamy macrophages and microglia. On the other hand MR expression is restricted to PVM in both normal and inflamed CNS tissue. We also show that PVM in the human brain express molecules involved in antigen recognition, antigen presentation and costimulation in normal brain tissue and MS lesions.

Materials and methods

Human Brain Tissue

Human brain tissue was obtained at autopsy from patients without neurological complications (controls) and from secondary progressive MS patients (for details see Table 1). Samples were obtained through the rapid (short post-mortem delay) autopsy regimen of the Netherlands Brain Bank in Amsterdam (coordinator Dr. R. Ravid), with approval from the Medical Ethical Committee of the VU Medical Center.

	Number	Age (years)	Sex	Disease duration (years)	PMD (hrs:min)
Control cases#	6	77.0	6F	-	8:53
MS lesions:					
Active#	3	59.0	3F	14.3	8:05
Chronic active#	2	66.5	1F 1M	30.5	10:25
Chronic inactive #	2	71.5	1F 1M	26.5	8:25
NAWM	1	45.0	F	14.0	10:55

Table 1: *Details of MS and normal control autopsy brain tissue*

Values are presented as the average between the subjects. F: female, M: male, PMD: post-mortem delay, Disease duration: mean disease duration since first clinical symptoms of MS, NAWM: normal appearing white matter.

Tissue samples (1.0-2.5 cm^3) from non-neurological control cases were obtained from the sub-cortical white matter or corpus callosum. For MS, the clinical diagnosis was confirmed neuropathologically (by Prof. Dr. P. Van der Valk and Dr. W. Kamphorst, Department of Pathology, VU Medical Center, Amsterdam). In all MS cases tissue samples were obtained after *ex-vivo* MRI scanning of the autopsy brain slices, as described previously (De Groot et al., 2001). Brain tissue samples were snap-frozen and stored in liquid nitrogen or fixed in 10% neutral buffered formalin for paraffin

embedding. Lesional staging was performed as described by Van der Valk and De Groot (Van der Valk and De Groot, 2000). In normal control white matter tissue no inflammatory cells were detected.

Immunohistochemistry

Serial 4 μm-thick frozen sections and paraffin sections from brain white matter were collected on Superfrost Plus glass slides. The frozen sections were air dried and fixed in acetone for 10 min at room temperature (RT). Paraffin sections were dried overnight at 37°C, deparaffinated and pre-treated with an antigen retrieval method by heating the slides in a microwave oven in citrate buffer (10 mM, pH 6.0) for 10 min (360 W). After cooling the sections were immersed in methanol containing 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. All wash steps were performed with 0.01M phosphate-buffered saline (PBS; pH 7.4) and the monoclonal antibodies (mAbs) were diluted in PBS containing 1% bovine serum albumin (BSA; Boehringer-Mannheim, Mannheim, Germany). For immunohistochemistry, sections were pre-incubated with either 2% normal rabbit serum (NRS; in case of mAbs) or 5% normal swine serum (NSS; in case of polyclonal antibodies; pAbs) for 10 min. The primary antibodies (for specifics see Table 2) were incubated for 1 hr at RT. After several wash steps the slides were incubated with biotinylated rabbit anti-mouse $F(ab')_2$ (1:500) or swine anti-rabbit IgG $F(ab')_2$ (1:300) (DAKO; Copenhagen, Denmark) as secondary pAbs for 30 min at RT. Sections were then incubated for 1 hr at RT with streptavidin-biotin-peroxidase complexes (strepABComplex HRP, DAKO; 1:200) as described previously (De Groot et al., 1999). Peroxidase activity was demonstrated with 0.5 mg/ml 3,3'-diaminobenzidine-tetrahydro-chloride (DAB; Sigma, ST Louis, MO) in PBS containing 0.03% H_2O_2 for 5 min to give a brown-coloured reaction product. Haematoxylin was used as counter stain. As a negative control, primary antibody was omitted or replaced by irrelevant isotype-matched murine mAb.

Double Immunofluorescence

Sections from control and MS white matter were collected on Superfrost Plus glass slides. After fixation, sections were double stained for EDHu-1 (Van den Heuvel et al., 1999) and several monoclonal antibodies (Table 2). Double immunofluorescence with antibodies of the same IgG subclass (Table 2) was performed with biotinylated EDHu-1 (produced in our own laboratory) to avoid cross staining of conjugates. Sections were acetone-fixed for 10 min and air-dried. Then the slides were pre-incubated with 2% normal goat serum (NGS) for 10 min. The first antibody (for dilutions see Table 2) was diluted in PBS containing 1% BSA and incubated for 1 hr. After several washing steps the first antibody was detected with goat anti-mouse $IgG1^{ALEXA\ 488}$ (1:400 in PBS containing 10% NGS and 1% BSA) (Molecular Probes Europe B.V., Leiden, The Netherlands) for 1 hr at RT. After rinsing in PBS the slides were incubated with biotinylated EDHu-1 for 1 hr at RT, which was detected with $streptavidin^{ALEXA594}$ (1:400, 1 hr at RT; Molecular Probes, Eugene, OR). Then, sections were incubated with Hoechst 33342 (1:5000, Molecular Probes, Oregon USA) for 1 min to detect the nuclei.

After washing the slides were mounted in Vectashield (Vector laboratories, Inc, Burlingame, CA, USA).

Double immunofluorescence with antibodies of different IgG subclasses was performed as follows. After fixation in acetone for 10 min and pre-incubation with 10% NGS for 10 min a mixture of the primary mAbs (Table II) was applied for 1 hr at RT. The mAbs were detected with a mixture of conjugates: biotinylated rabbit-anti-mouse IgG2b, biotinylated rabbit-anti-mouse IgG2a or biotinylated rabbit-anti-mouse IgM (1:400, 1:2000 or 1:400) (Zymed, San Francisco, CA) together with goat-anti-mouse $IgG1^{ALEXA\ 594}$ (1:400 in PBS containing 10% NRS respectively 10% NGS and 1% BSA) (Molecular Probes Europe B.V., Leiden, The Netherlands). After rinsing in PBS the slides were incubated with streptavidin-FITC (1:400, Vector, Burlingame, CA) for 1 hr. Sections were washed and then incubated with Hoechst for 1 min and mounted in Vectashield.

As negative controls, primary antibodies were omitted and replaced by irrelevant isotype-matched controls throughout. When sections showed auto fluorescence (Romijn et al., 1999), the staining protocol was extended by incubating the sections, after the Hoechst staining, in Sudan Black B solution (0.3% Sudan Black B (Merck, Darmstadt, Germany) in 70% ethanol at RT in the dark for 2 hr and leaving it standing overnight, followed by filtration) for 20-60 sec, washing the sections twice with PBS and finally mounting the sections in glycerol/50mM Tris.

All sections were analyzed with a Nikon Eclipse E800 fluorescence microscope; the recordings were made with a Nikon DXM 1200 camera and processed using Nikon ACT-1 software.

Antibody	Clone	Isotype	Cellular specificity	Dilution
EDHu-1 [1]	-	Mouse IgG1	Human CD163, Resident macrophages	1:200
CD86 (B7-2) [2]	IT2.2	Mouse IgG2b	Costimulatory signal	1:100
CD80 (B7-1) [2]	BB1	Mouse IgM	Costimulatory signal	1:100
CD68 [3]	KP1	Mouse IgG1	Macrophages/microglia cells	1:800
CD45 (LCA) [3]	-	Mouse IgG1	Leukocytes	1:50
CD40 [2]	5C3	Mouse IgG1	Receptor for costimulatory signals	1:400
CD8	OKT8	Mouse IgG2b	Cytotoxic T cells	1:10
CD4 [1]	BF5	Mouse IgG1	T-helper cells	1:100
SMA [3]	-	Poly IgG	Pericytes	1:200
Laminin [3]	-	Poly IgG	Basal lamina	1:100 (frozen) 1:50 (paraffin)
HLA-ABC [2]	-	Mouse IgG1	MHC class I	1:100
MHC class II [2]	9.3F10	Mouse IgG2a	MHC class II	1:10
DC-SIGN [6]	AZN-D1	Mouse IgG1	Dendritic cells	1:1
MR [7]	3.29B1	Mouse IgG1	Dendritic cells	1:1

Table 2: *Summary of used antibodies*

[1] Developed in our own laboratory (Van den Heuvel et al., 1999) and commercially available from Serotec, Oxfordshire, UK.
[2] BD Bioscience Pharmingen, San Diego, CA.
[3] DAKO, Copenhagen, Denmark.
[4] American Type Culture Collection (ATCC), Rockville, MD.
[5] Molecular probes, Eugene, OR.
[6] Kindly provided by Prof. Dr. Y. van Kooyk (Geijtenbeek et al., 2000) from our department.
[7] Kindly provided by Dr. Cella (Washington University School of Medicine, St. Louis, MO).

Results

Identification of PVM in the normal human CNS

In normal brain tissue, obtained at autopsy, CD163 expression was observed on a typical population of perivascular cells, reminiscent of PVM (Figure 1A), as well as on meningeal macrophages (data not shown). The CD163 staining pattern resembled that of PVM observed in rat brain tissue using the anti-rat CD163 reactive mAb ED2 (Dijkstra et al., 1985; Graeber et al., 1989). Double immunofluorescence with various markers was performed to further characterize the CD163-positive cells in the CNS perivascular space (Figures 1B-D). Staining for laminin (Figure 1B), which is a major constituent of the basal membrane of the BBB endothelial cells, revealed that the CD163-positive cells were located within the Virchow Robin space and outside the vascular basement membrane. Furthermore, all CD163-positive cells were positive for CD68 (a macrophage marker, Figure 1C) and negative for SMA (smooth muscle actin, Figure 1D).

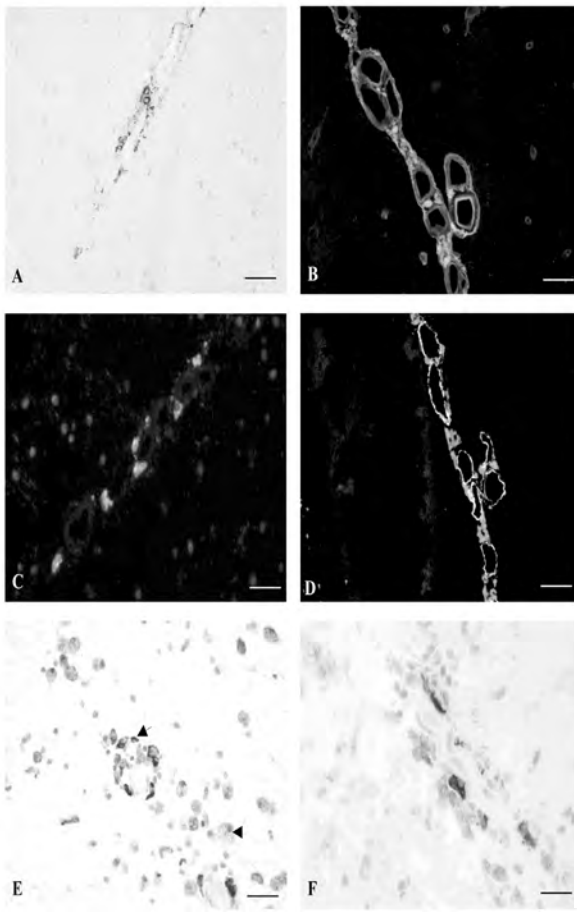

Figure 1: *CD163 expression normal and MS lesion white matter*

For a full colour picture, see Appendix. **A:** Control white matter, frozen section stained with mAb EDHu-1 against CD163. PVM are CD163-positive and have an elongated shape around a medium-sized blood vessel. **B-D:** Control brain, cerebral white matter, frozen section stained with double immunofluorescence for CD163 (red) and laminin (green; **B**), CD68 (green; **C**), smooth muscle actin (green; **D**). CD163-expressing cells are CD68-positive macrophages. The PVM lie between the laminin-positive endothelial and glial basement membranes and are SMA-negative. **E**: Active lesion, frozen section, mAb EDHu-1 against CD163. Parenchymal macrophages (arrowhead, foam cells) are weakly positive. PVM (arrow) near blood vessels at the lesion edge are strongly positive. **F:** Chronic active lesion, paraffin embedded section. On the right the inactive centre of the lesion is seen, with hardly any positive cells. The predominant cell type is the strong staining; rounded PVM, but weaker staining foam cells and some microglial cells are also visible. Scale bar = 100 (A, E) and 50 um (B, C, D, F).

This indicates that the CD163-positive cells were indeed macrophages and not pericytes. CD163-positive cells displayed weak expression of the leukocyte common antigen (LCA, CD45; data not shown). In normal brain ramified microglial cells did not express CD163. Overall these results clearly demonstrate that CD163 can be used to identify PVM in the normal CNS.

	Perivascular macrophages	Foamy macrophages	Infiltrating monocytes	Microglia
Control tissue (n = 6)	++	a	a	–
NAWM	++	a	+*	–
Active lesion (n = 3)	+++	+*	+*	±
Chronic active lesion (n = 2)	+++	+*	+*	±
Chronic inactive lesion (n = 2)	+	a	a	–

Table 3: *Distribution of CD163 expression in the human CNS*

a: cell type absent, – No CD163 staining present, ± Occasional CD163 staining present, + weak CD163 staining present, ++ strong CD163 staining, +++ very strong staining and an increase in number of CD163-positive cells. *10-30% of all blood monocytes are positive for CD163.

PVM in MS brain tissue

CD163 expression was studied in different types of MS lesions (Table 3, Figures 1E/F). In normal appearing white matter (NAWM), adjacent to MS lesions, CD163 expression was restricted to PVM as seen in control white matter and vessels were observed to have more, larger and rounder CD163-positive PVM than in normal brain tissue. In active, chronic active and chronic inactive lesions CD163-positive PVM could still be clearly distinguished: they were increased in number and CD163 expression on individual PVM seemed to be upregulated (Figures 1E/F). However, the majority of inflammatory macrophages associated with MS lesions were also CD163-positive, albeit weakly. Active lesions were characterized by the presence of foamy macrophages, which were positive for CD163 (Figure 1E). In active lesions and in chronic active lesions some ramified microglial cells were weakly CD163-positive.

PVM express molecules involved in antigen recognition and presentation

A. Healthy CNS

The role of PVM as APC of the CNS is still controversial. It is clear that PVM in rodents express at least MHC class II molecules but the situation in human however has not been thoroughly studied. We studied the expression of several pathogen-associated molecular pattern (PAMP) recognition receptors, including MR and DC-SIGN that could potentially be related to antigen recognition and uptake by PVM. Both these molecules were restricted to PVM in normal control brain tissue (Figures 2B/C).

In order to investigate the antigen-presenting potential of PVM in the human brain we studied the expression of MHC class II and costimulatory molecules by means of double immunofluorescence (a summary of the results is given in Table 4). All CD163-positive PVM co-expressed MHC class II (Figure 2A).

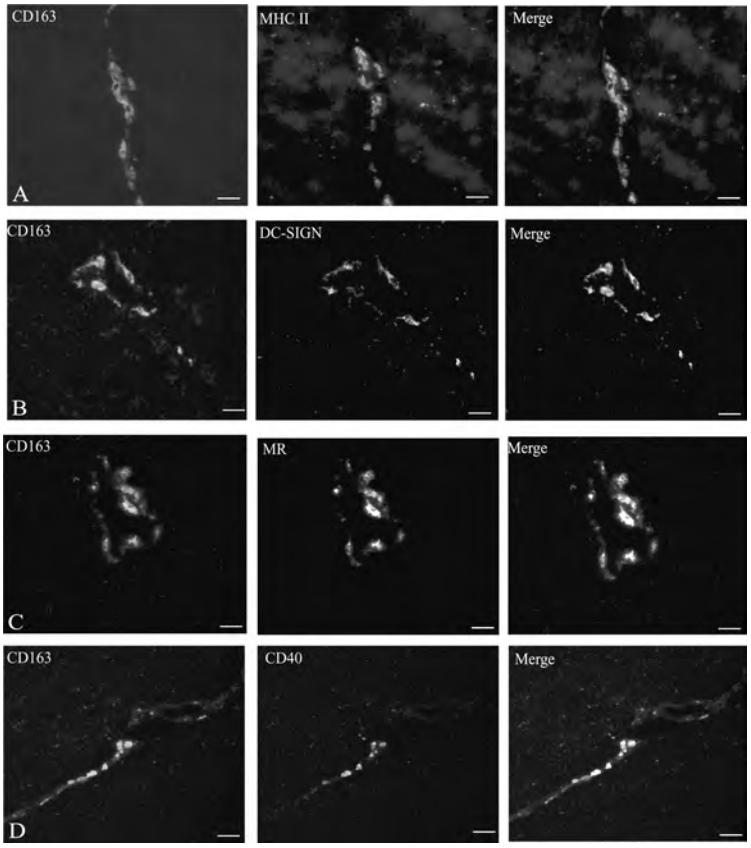

Figure 2: *Double immunofluorescence for CD163 and other molecules in normal white matter.*
For a full colour picture see Appendix. **A:** Control cerebral white matter, double immunofluorescence for MHC class II (green) and CD163 (red). CD163-positive macrophages are positive for MHC class II. **B:** Control cerebral white matter, double immunofluorescence for DC-SIGN (green) and CD163 (red). There is colocalization of CD163 and DC-SIGN on a subpopulation of PVM**. C:** Control cerebral white matter, double immunofluorescence for MR (green) and CD163 (red). All CD163-positive PVM express MR. **D:** Control cerebral white matter, double immunofluorescence for CD40 (green) and CD163 (red). A subpopulation of CD163-positive PVM shows staining for CD40.Scale bar = 50 µm.

Optimal activation of T cell responses requires additional interactions between costimulatory molecules expressed on APC (such as B7-1, B7-2 and CD40) and specific T cell receptors. In normal control brain tissue no B7-1-positive cells were detected, whereas B7-2 was expressed by a subset of CD163-positive PVM. CD40, a molecule that acts as a costimulatory signal for activated T cells expressing CD40 ligand, was also restricted to PVM in normal control tissue (Figure 2D). Notably, B7-2, CD40 and DC-SIGN were expressed on a subpopulation of PVM that were round in shape and had a swollen morphology, while the more elongated and slim PVM did not express these molecules (Figures 2B/D). On the other hand MR was observed on all PVM (Figure 2C). Triple immunofluorescence for CD163, DC-SIGN and CD40 was performed (data not

shown): this provided evidence for several phenotypically distinguishable PVM subpopulations, including CD163-positive DCSIGN- and CD40-negative, CD163- DC-SIGN-positive and CD40-negative, and CD163- DCSIGN -CD40-positive.

B. Multiple sclerosis

In MS lesions there was an upregulation of molecules involved in antigen recognition, antigen presentation and costimulation, which extended beyond PVM to include foamy macrophages and microglia, with the exception of MR (Table 4, Figure 3A). Thus foamy macrophages present in active and chronic active lesions were positive for CD163 (Figure 1E), DC-SIGN (Figure 3B), CD40 (Figure 3C), B7-1 and B7-2. We observed a close association of PVM and T lymphocytes in perivascular infiltrates consistent with local interaction between these two cell types (Figure 3D).

It was interesting to note that, in contrast to CD163, MR expression in both normal and inflamed CNS tissue was restricted to PVM (Figure 3A), which also corresponds to recent findings in mice (Galea et al., 2004).

	PVM	Foamy macrophages	Microglia
MHC class II	++	++	-/+
CD163	+++	+	-/+
CD80 (B7-1)	+*	+	-
CD86 (B7-2)	+*	+	-
CD40	+*	+	-
CD68	+++	+++	-/+
DC-SIGN	+*	+++	-
MR	+++	-	-

Table 4: *Expression of molecules important for antigen recognition, antigen presentation and costimulation by macrophages in MS*

*A subpopulation of perivascular macrophages is positive for this marker. – No staining present, ± Occasional staining present, + weak staining present, ++ strong staining, +++ very strong staining.

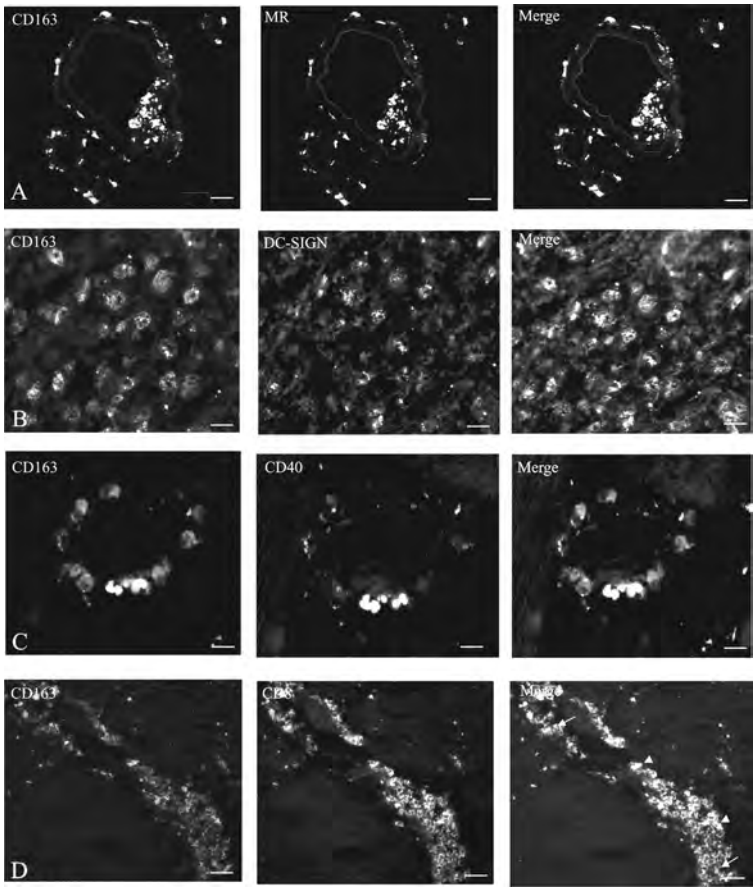

Figure 3: *Double immunofluorescence for CD163 and other molecules in MS lesion white matter.*

For a full colour picture see Appendix. **A-D**; Double immunofluorescence for CD163 and other molecules in MS white matter **A.** Active MS lesion, double immunofluorescence for MR (green) and CD163 (red). Foamy macrophages do not express MR but do express low levels of CD163 (not shown in this picture). **B.** Active MS lesion stained for DC-SIGN (green) and CD163 (red). Foamy macrophages within the lesion are weakly positive for CD163 and also for DC-SIGN. **C.** Active MS lesion, double immunofluorescence for CD40 (green) and CD163 (red). Subpopulations of CD163-positive PVM express CD40. **D.** Chronic active lesion stained for CD8 (green) and CD163 (red). There is close apposition (arrow) of the green lymphocytes with the red PVM and an occasionally overlap (arrowhead) of CD8 and CD163 in an infiltrated vessel near the lesion site. Scale bar = 100 um (A and D), scale bar = 50 um (B and C).

Discussion

In this study we set out to explore the function of PVM in the human CNS by investigating their expression of molecules with known function in antigen recognition, presentation and costimulation. However, we first had to establish a marker for this specific subpopulation of CNS macrophages in human tissue. We therefore validated the use of the anti-human CD163 monoclonal antibody EDHu-1 as a marker for PVM. In normal brain, CD163 expression was restricted to PVM and meningeal macrophages, whereas microglia, inflammatory macrophages and pericytes were CD163-negative (Table 3).

Under inflammatory conditions, like MS, increased numbers of strongly CD163-positive PVM were found (Table 3). However, foamy macrophages containing myelin debris in active MS lesions were also CD163-positive, but the level of expression was clearly lower than that of PVM, and the typical shape of foamy macrophages made distinction from PVM fairly straightforward. In addition, microglial cells within or directly next to the lesion occasionally expressed CD163. CD163 expression in the rat is restricted to PVM in both inflammatory (e.g. EAE) and non-inflammatory conditions. The discrepancy between rat and human CD163 expression in the CNS might be related to the fact that human monocytes are 10-30% positive for CD163 (Hogger et al., 1998), whereas rat monocytes are CD163-negative (Dijkstra et al., 1985). During the course of investigating the PVM phenotype we observed that in addition to CD163 staining on PVM also foamy macrophages are positive for CD163 in MS lesion tissue, whereas MR stains only PVM in inflammatory and non-inflammatory tissues.

PVM reside at the abluminal side of the BBB and are therefore strategically located to exert antigen recognition and scavenger functions. Indeed we have shown that human PVM express CD163, MR and DC-SIGN, which is compatible with such roles. CD163 is a hemoglobin-haptoglobin scavenger receptor (Kristiansen et al., 2001), while MR and DC-SIGN are PAMP receptors involved in antigen recognition and uptake (Linehan et al., 2000). Cross-linking of CD163 by EDHu-1 results in protein kinase C dependent macrophage activation and cytokine production (Van den Heuvel et al., 1999; Kristiansen et al., 2001; Ritter et al., 2001), which may contribute to the establishment or regulation of an inflammatory response in the perivascular space.. This is in line with earlier reports that have suggested a role for PVM in antigen presentation (Hickey and Kimura, 1988; Polfliet et al., 2002). Recent experiments in our lab have alerted us to the presence of FcγRI (CD64) and FcγRII (CD32) on a subpopulation of PVM (unpublished data). FcγR-induced phagocytosis also plays a role in antigen presentation and amplification of the immune response (Aderem and Underhill, 1999). In addition to the expression of molecules mentioned above, evidence supporting a scavenger role of PVM comes from functional animal studies in which injected exogenous macromolecules were selectively taken up by PVM (Kida et al., 1993; Mato et al., 1996; Angelov et al., 1996).

Besides tools for recognition and uptake of antigen, we have also shown that PVM possess the appropriate tools to present antigen. PVM were positive for MHC Class II and several costimulatory molecules (like CD80, CD86 and CD40). Indeed PVM were

observed lying in close vicinity to CD4-positive and CD8-positive lymphocytes, which is consistent with a local interaction between PVM and T lymphocytes. These findings in the human CNS are in line with earlier rodent studies that indicate a role for PVM in antigen presentation (Hickey and Kimura, 1988; Polfliet et al., 2002).

A not yet described of feature of PVM is that there appear to be different subpopulations of these cells expressing different receptors and with a different morphology. One might speculate that these subpopulations might indicate several differentiation phases of PVM with different functions. Further studies should be performed to further characterize these subpopulations with a possible difference in function and morphology since this was not in the scope of this study.

Although our results support a role of PVM as APC perhaps locally in the CNS, migration of PVM to another site following antigen presentation seems also possible. A possibility is that PVM migrate to the nearest lymph node to present antigen there. Alternatively, CNS antigen may bypass PVM completely by draining along perivascular spaces to the periphery where it may be taken up by local professional antigen-presenting cells in peripheral lymphoid organs.

In summary, we have shown that the cell surface molecules CD163 and MR can be employed to identify human PVM in both normal and inflamed brain tissue. Furthermore, our findings indicate that PVM, which occupy a strategic location at the BBB, are equipped to recognize antigen, process it and present it to T cells, supporting a possible role in the regulation of perivascular inflammation in the human CNS.

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5.

In vivo detection of myelin proteins in cervical lymph nodes of MS patients using ultrasound guided fine-needle aspiration cytology

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Abstract

Cervical lymph nodes (CLN) have been described to be the first lymphoid draining site of the brain. In this study we used ultrasound guided fine needle aspiration cytology (USgFNAC) to obtain cells, *in vivo*, from non-enlarged CLN of Multiple Sclerosis (MS) patients and healthy controls (HC), and investigated whether myelin proteins could be detected. Macrophages containing myelin basic protein (MBP) and proteolipid protein (PLP) were found in CLN of all MS patients, whereas these could only be detected in a minority of HC. This novel approach allows investigation of the first draining site of the brain for *in vivo* analysis of the immune regulation of MS.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) characterized by a pathology associated with invasion of the brain by T cells and macrophages through the blood-brain barrier, causing damage to myelin sheaths and axons (Lassmann, 1998). A recent study postulates heterogeneity in the immunopathogenesis of MS and suggests that, in at least some subtypes, an immune response against myelin proteins plays a role (Lucchinetti et al., 2000; Berger et al., 2003).

In the CNS local immune responses can be a result of reactivation of antigen-specific cells that were primed in a lymphoid organ. The cervical lymph nodes (CLN) are in fact the first draining site of the brain and therefore reflect the first site of encounter between myelin antigens and naive T lymphocytes (Weller, 1998). Non-human primates with experimental autoimmune encephalomyelitis (EAE) showed the presence of myelin components in cells expressing dendritic cell and macrophage markers as well as HLA-II and costimulatory molecules in the CLN. These observations were confirmed in post-mortem CLN of 2 MS patients (De Vos et al., 2002). Previously, it has been shown that injecting a soluble antigen, albumin, in the grey matter of the rat brain resulted in antibody formation in the CLN (Harling-Berg et al., 1989). On the one hand, removal of the CLN from EAE rats reduced the severity of cerebral EAE by 40%, due to the lack of T cell priming (Phillips et al., 1997). On the other hand, CLN form the site of tolerance induction after nasal administration of an antigen through the induction of regulatory T cells in the CLN (Unger et al., 2003). This could be due to the specialized microenvironment of the CLN (Wolvers et al., 1999) and may thus also hold true for brain-derived antigens.

Collectively, these findings suggest that activation of naive B and T cells in the CLN is mediated by antigen-presenting cells that contain brain-derived antigens and may induce regulatory responses, which balance disease promotion and tolerance induction. We have shown in autopsy material that the myelin auto-antigens, formed in MS by the breakdown of the myelin sheath of axons, reach the CLN. Here, we provide a new approach to address this hypothesis *in vivo* in MS patients by showing that USgFNAC can be used to sample non-enlarged lymph nodes of MS patients and healthy controls (HC) and that myelin antigens can be detected *in vivo* in these CLN.

2. Materials and Methods

10 patients with clinically definite MS and 10 age and gender matched HC were recruited. HC had a mean (± SD) age of 36.6 (± 11.5) years. MS patients had a mean age of 37.3 (± 10.3) years, a median disease duration of 6.3 (4.8 – 15.7) years, and a median Expanded Disability Status Scale (EDSS) of 3.5 (2.4 – 6.0) (Table 1). None of the subjects experienced an infectious illness within 30 days prior to participation. The Medical Ethics Committee of our hospital approved the study and written informed consent was obtained.

For the detection and collection of samples from the CLN we used ultrasound-guided fine-needle aspiration (USgFNAC) (Van den Brekel et al., 1991). USgFNAC combines the high sensitivity of ultrasound (US) to detect non-palpable nodes with the high specificity of fine needle aspiration cytology. All levels of both sides of the neck were fully examined for the presence of lymph nodes. The minimal axial diameter of lymph nodes visible at US was measured. The largest and most cranial lymph node(s) in one or both sides of the neck were punctured under US guidance. The syringe containing the cell aspirate was flushed several times in Dulbecco's Modified Eagle medium (DMEM, Invitrogen Corporation, Breda, The Netherlands). The mean (± SD) total cell yield per patient after erythrocyte lysis was 2.7 10^6 (± 3.9 10^6) cells.

Cytocentrifuge preparations were fixed with 4% paraformaldehyde and stained for myelin specific proteins using monoclonal antibodies mouse-anti-human MBP22 (IgG2b, gift from prof. N. Groome, Oxford Brookes University, Oxford, UK) (Groome et al., 1988) and mouse-anti-human PLP (IgG2a, Instruchemie, Delfzijl, The Netherlands) or for the T cell specific marker mouse-anti-human CD3 (Leu4; American Type Culture Collection (ATCC), Rockville, MD). Staining was performed by immunoperoxidase (IP) using an avidin-biotin technique with a biotinylated rabbit-anti-mouse $F[ab']_2$ (DAKO; Copenhagen, Denmark) and by double immunofluorescence staining (IF) with the macrophage marker mouse-anti-human CD68 (KP1, IgG1, DAKO, Copenhagen, Denmark). Secondary antibodies used for IF were rabbit-anti-mouse Ig $F[ab']_2^{FITC}$ (DAKO, Copenhagen, Denmark) and mouse $IgG1^{ALEXA488}$ (Molecular Probes, Eugene, OR) and for IP rabbit-anti-mouse IgG $F[ab']_2$ (Jackson ImmunoResearch Laboratories Inc., Westgrove, PA).

Control stainings included omission of the first antibody and replacing it by an irrelevant isotype matched control. Staining was judged positive if macrophages were observed that contained MBP or PLP. Two independent observers blinded for the clinical data evaluated the stainings.

3. Results

In all subjects at least one lymph node with a minimal axial diameter of 3.0 mm was found in the subdigastric region. Overall the punctures were well tolerated. A few subjects complained of a small hematoma or pain, which was always minor and transient. The analysis focused on the presence of macrophages containing MBP and PLP using both IF double stainings and IP, resulting in a total of four stainings per subject (MBP-IF, MBP-IP, PLP-IF, PLP-IP). Data are summarized in Table 1. Four subjects (2 HC; 2 MS patients) had to be excluded due to insufficient technical quality of the cytocentrifuge preparations. Overall, a large variation in macrophages between subjects was observed. In 3 HC no macrophages could be observed by IF staining for the macrophage marker CD68, although CD3-positive T cells were present. This was not related to the size of the CLN.

	Gender	MS type	IFN-β	Disease Duration	EDSS	Minimal axial ∅ of punctured lymph nodes in mm. (nr. of punctures) *subdigastric region right*	*subdigastric region left*	MBP IP	PLP IP	MBP IF	PLP IF
HD 1	M					5·5 (1)	6·3 (1)	–	–	–	–
HD 2	F					6·0 (1)	5·5 (1)	–	–	–	–
HD 3	F					5·4 (2)		–	–	–	–
HD 4	F					4·8 (1)	3·4 (1)	–	–	–	–
HD 5	M					5·1 (1)	5·6 (1)	Excl	Excl	Excl	Excl
HD 6	F					3·8 (1) 3·4 (1)		–	–	–	–
HD 7	M					6·3 (1)	4·4 (1)	–	–	–	–
HD 8	F						6·0 (2)	–	–	–	–
HD 9	F					5·1 (2)	4·4 (1)	Excl	Excl	Excl	Excl
HD 10	F					6·2 (1)	6·8 (2)	–	–	–	+
MS 1	M	SP	No	27·1	3·0	5·6 (1)	5·4 (1)	–	–	–	+
MS 2	F	RR	Yes	14·0	2·5	4·1 (3)		–	x	–	+
MS 3	M	RR	Yes	2·0	2·0	6·3 (1)	6·4 (1)	–	–	–	+
MS 4	F	SP	Yes	6·6	4·0	6·7 (1)	8·7 (1)	–	–	–	–
MS 5	F	RR	Yes	6·0	1·5		3·9 (3)	–	–	+	+
MS 6	F	RR	No	3·5	6·0		4·6 (2)	Excl	Excl	Excl	Excl
MS 7	M	RR	Yes	5·3	3·5	6·9 (1)	5.0 (1)	–	–	+	+
MS 8	F	SP	No	20·7	8·0	3·9 (1)	4·0 (1)	Excl	Excl	Excl	Excl
MS 9	M	SP	Yes	13·4	6·0	7·3 (1)	6·5 (1)	–	+	–	+
MS 10	F	RR	Yes	5·5	3·5		5·9 (2)	–	–	–	+

Table 1: *Subject characteristics*

M: male, F: female, RR: relapsing remitting MS, SP: secondary progressive MS, IFN: interferon treatment, EDSS: Expanded Disability Status Scale. Age and disease duration are given in years. IP: immunoperoxidase staining, IF: double immunofluorescence staining. +: positive staining, –: no staining. Excl: Excluded from the study due to poor technical quality of the cytospin preparations, x: not performed due to lack of material. In control subjects 2,3, and 4 no macrophages could be observed, but CD3-positive cells were present.

In CLN of HC, macrophages containing MBP were observed in one out of 8 subjects and macrophages containing PLP also in one out of 8 subjects (not the same subject) (Figure 1A/B). In contrast, in CLN of 6 out of 8 MS patients MBP containing macrophages were observed and in CLN of all MS patients under study (8 out of 8) macrophages staining for PLP were observed (Figures 1C-F).

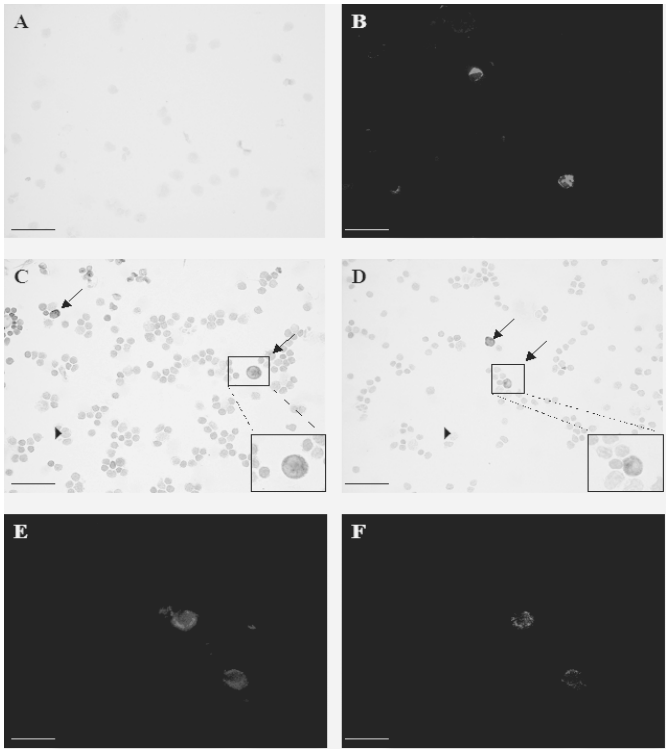

Figure 1: *Immunohistochemical and immunofluorescence staining of CLN aspirates*

For a full colour picture see Appendix. **A.** Immunoperoxidase staining of the CLN material of a HC (nr. 10) stained for MBP. In blue the nuclei of the cells are visible by haematoxylin counterstaining. No MBP staining observed. **B.** CLN material of a healthy control subject (nr. 8) stained for MBP (green) and CD68 (red) by immunofluorescence. Several macrophages (red) are visible whereas no MBP staining (green) can been observed. **C/D.** CLN material of a MS patient (nr. 7) stained by immunoperoxidase for MBP (C) or PLP (D). MBP or PLP positive cells are indicated with an arrow, negative macrophages with an arrowhead**. E/F.** CLN material of an MS patient (nr.7) stained for MBP (green) and CD68 (red) by immunofluorescence. Several macrophages (red) are visible containing MBP (green) (scale bar = 50 μm).

4. Discussion

In this study we show that by means of USgFNAC, a minimally invasive procedure, it is possible to obtain material from the non-enlarged CLN of MS patients and HC. Furthermore, we describe the presence of myelin antigens in the CLN of *in vivo* MS patients and HC. Macrophages containing myelin antigens could be observed in the CLN of all MS patients included in this study, whereas in CLN of HC only in 2 out of 8 cases myelin protein containing macrophages were found. These results show that myelin antigens can reach the secondary lymphoid organs, where priming of T cells targeting the brain can occur. It remains to be established how these myelin antigens reach the CLN; this may either be by transport within dendritic cells or macrophages or as a free particles.

Expression of myelin proteins within activated macrophages can be used to assess the staging of the demyelinating lesions. Presence of myelin oligodendrocyte glycoprotein (MOG) and myelin associated oligodendrocyte glycoprotein (MAG) in macrophages indicates a very recent demyelination (within the previous 3 days). Whereas immunoreactivity for PLP and MBP indicates lesional activity within the previous 10 days (Bruck et al., 1995). The observed differences in degradation time could be caused by a difference in abundance or a difference in protease sensitivity of these proteins. Our finding that PLP was detected in the CLN of most MS patients and MBP less frequent might be due to the fact that MBP is more sensitive for lysosomal acid proteases and neutral proteases (Shields et al., 1999). The time course of myelin protein digestion in macrophages *in vitro* indicates that the macrophages containing MBP or PLP in the CLN have ingested myelin within one week before USgFNAC, and therefore may reflect recent disease activity. In controls this may reflect the aspects of myelin membrane turnover, which supposedly is at a considerably lower rate (Ando et al., 2003). Due to the large differences in macrophage-numbers between the subjects and the relatively small amount of material obtained from the aspirations, quantitative studies are not feasible with the current methodology.

Future studies, involving larger numbers of patients, may shed light on the immune regulatory consequences of the presence of myelin protein in the CLN. First it has to be established if myelin is transported to the CLN within macrophages or dendritic cells. Furthermore, it would be interesting to know if neuronal degradation products also reach the CLN. Another important question that needs to be elucidated is which cell type presents neuronal and/or myelin antigen to naive T cells. This could determine if an immune response is raised and what the nature is of such an immune response, with respect to the Th1/Th2 balance. The properties of the antigen-presenting cells together with the microenvironment of the CLN also determines, whether tolerance is induced by the propagation of regulatory T cells (Wolvers et al., 1999; Unger et al., 2003). In addition it is now possible to investigate whether the pattern of findings in the CLN is related to the course or activity of the disease and to detect the different types of myelin breakdown products present in the CLN.

In conclusion, the novel approach presented in this study allows *in vivo* investigation of the human CLN, which as first lymphoid draining station of the brain will reveal important information on the immune regulation of MS.

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6.

Proteolytic shedding of the macrophage scavenger receptor CD163 in Multiple Sclerosis

Submitted

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Abstract

The scavenger receptor CD163 is selectively expressed on tissue macrophages and human monocytes. CD163 has been implicated to play a role in the clearance of hemoglobin and in the regulation of cytokine production by macrophages. Membrane CD163 can be cleaved by matrix metalloproteinases (MMP) resulting in soluble CD163 (sCD163). In the present report the shedding of CD163 was investigated in Multiple Sclerosis (MS). An upregulation of sCD163 and a down regulation of membrane CD163 in MS patients compared to healthy controls was observed. The levels of sCD163 correlated with plasma MMP-9 levels in controls, but not in MS patients. Moreover, evidence was obtained for CD163-cleaving MMP activity in plasma of MS patients. Finally, the increased proteolytic shedding of CD163 correlated to reduced levels of circulating inflammatory cytokines. Collectively, our results provide evidence for proteolytic shedding of CD163 in MS and suggest a possible link to cytokine production.

1. Introduction

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) and is characterized by the presence of sclerotic lesions or plaques, scattered throughout the brain (Lucchinetti et al., 2000; De Groot et al., 2001). Early events in the development of MS lesions are increased permeability of the blood–brain barrier (BBB) and the formation of cellular infiltrates, consisting of monocytes and lymphocytes. In a previous study, an increased number of perivascular macrophages and foamy macrophages, expressing the scavenger receptor CD163, was observed within various stages of MS lesions (Chapter 4) (Boven et al., 2006).

CD163 is a member of the scavenger receptor cysteine-rich (SRCR) family class B and is expressed exclusively on monocytes and macrophages (Van den Heuvel et al., 1999) (Chapter 4). CD163 positive macrophages are found during the healing phase of inflammation but also in chronic inflammatory disease (Moestrup and Moller, 2004). CD163 functions as an endocytic receptor for hemoglobin-haptoglobin (Hb-Hp) complexes and as such is proposed to mediate the clearance of free Hb from the circulation (Kristiansen et al., 2001). There is evidence to support an immune-modulatory role of CD163. In particular, the cross-linking of CD163, either by antibodies (Van den Heuvel et al., 1999) or Hb-Hp complexes (Kristiansen et al., 2001) results in a potent cytokine response.

Mature tissue macrophages express high levels of CD163, for instance Kuppfer cells in the liver, red pulp macrophages in the spleen, cortical macrophages in the thymus, and PVM in the brain (Van den Heuvel et al., 1999).

Membrane bound CD163 can be actively shed from macrophages by a matrix metalloproteinase (MMP)-dependent release mechanism in response to inflammatory stimuli (Droste et al., 1999; Timmermann and Hogger, 2005). Cleavage removes the known ligand-binding site present in SRCR domain 3 (Madsen et al., 2004), and therefore the ability to induce CD163 cross-linking. Furthermore, there is some evidence that sCD163 may also have a direct immune-modulatory capacity (Hogger and Sorg, 2001; Frings et al., 2002; Baeten et al., 2004). Levels of plasma sCD163 are increased in a number of pathological conditions, including sepsis, pneumonia, rheumatoid arthritis (RA), myeloid leukemia and Gaucher disease (Moller et al., 2002a; Moller et al., 2004; Weiss and Schneider, 2006; Moller et al., 2006). It appears that elevated sCD163 levels are associated with both macrophage activation (Hintz et al., 2002; Baeten et al., 2004), as well as proliferation (Hintz et al., 2002; Moller et al., 2002a; Moller et al., 2004; Baeten et al., 2004).

In the present study we have investigated the expression of membrane CD163 on monocytes and the levels of sCD163 in MS patients. Our results provide evidence for an enhanced proteolytic shedding of CD163 in MS, which is associated with decreased plasma pro-inflammatory cytokine (IL-6 and IL-12p40) levels. Furthermore, we identify a selective MMP-like proteolytic activity in the plasma of MS patients that can mediate CD163 cleavage.

2. Materials and Methods

Patients and PBMC isolation

For sCD163 analysis samples from 473 well-documented MS patients and 29 healthy controls (HC) were used. Patients of different clinical subtypes types of MS (relapsing and progressive) were present in this database. Mean age of all MS patients was 43 years (range 18-84) and the mean age of the controls was 40 years (range 29-62). The male to female ratio in the MS patients was 164/309 and in the HC 14/15. An Expanded Disability Status Scale (EDSS) score was obtained at the time of sampling from all MS patients. The mean EDSS score was 4.1 (1.0 - 9.0) and the disease duration among the MS patients was: 7.7 (0.1-27) years. To extend our studies on sCD163 in MS in depth we selected 34 additional MS patients and 10 age-matched HC for further studies. HC were recruited mainly from personnel working in our institute. Blood was collected between 11.00 a.m. and 15.00 p.m. Patient characteristics of this additional group are listed in Table 1. The medical ethics committee of our hospital approved the study.

	MS	HC
Number	34	10
Male/Female	10/24	6/4
Age	43.5 ±11.7	45.9 ± 11.6
Disease duration	10.6 ± 7.8	-
Relapsing/Progressive MS	23/11	-
EDSS	4.1 ± 1.8	-

Table 1: *Patient characteristics*

Study procedure

Soluble CD163 was studied by sandwich ELISA and CD163 membrane expression by flow cytometry. Briefly, 30 ml of heparinized venous blood was centrifuged at 2000 rpm at room temperature (RT). Plasma was collected for ELISA and the cell pellet was resuspended in phosphate buffered saline (PBS). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation over Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) at 170*g* for 45 min. PBMC were stored at –80°C until flow cytometry was performed.

sCD163 ELISA

Soluble CD163 was detected in the plasma of MS patients as previously described (Moller et al., 2002b). Briefly, polyclonal rabbit anti-CD163 (4 mg/l) (Kristiansen et al., 2001) was used for coating. After washing, 100µl of sample (diluted 1:50 in PBS with albumin, pH 7.2) was added and incubated for 1 hr. Samples were washed and then incubated with 100 µl of mouse monoclonal anti-CD163 (GHI/61, diluted 1:500, Research Diagnostics) for 1 hr. Following washing, wells were incubated with peroxidase-conjugated goat anti-mouse (1:4000, DAKO; Copenhagen, Denmark) for 1 hr. The wells were washed and 100µl of H_2O_2/1,2-phenylenediamine dihydrochloride

substrate solution was added. After 15 min, 50µl of 1M H_2SO_4 was added, and plates were read at 492/620nm. Control samples and standards of purified CD163 were co-analysed in each run.

Flow cytometry

Flow cytometric analysis of CD163 expression on PBMC was performed by co-staining with the monocytic marker CD14. Dual-colour immunofluorescence was performed on thawed PBMC using the following monoclonal antibodies: anti-human CD163 (EDHu-1 (Van den Heuvel et al., 1999), produced at our own laboratory and commercially available via Serotec, Oxfordshire, UK) and phycoerythrin (PE)-conjugated anti-human CD14 (BD Bioscience Pharmingen, San Diego, CA). Cells were stained with EDHu-1 (10 µg/ml) for 1 hr at 4°C in PBS-0.1% bovine serum albumin (BSA; Boehringer-Mannheim, Mannheim, Germany), and then washed. Subsequently, cells were stained with FITC-conjugated-rabbit anti mouse IgG (1:300; DAKO; Copenhagen, Denmark) for 45 min. After washing, cells were incubated with 2% normal mouse serum and then stained with CD14-PE (1:400) and incubated in the dark for 1 hr at 4°C in PBS-0.1% BSA. After washing cells were resuspended in PBS-0.1% BSA supplemented with 1% paraformaldehyde and fluorescence intensity was determined the following day using a FACS-calibur flow cytometer (Becton and Dickinson, San Jose, CA, USA). A minimum of 100,000 events was collected in list mode on FACS-calibur software. Monocytes were gated based on forward-sideward scatter profiles and CD14 expression was used to verify whether the gated cells were indeed monocytes.

MMP-2 and MMP-9 activity assay

MMP-2 and MMP-9 were measured with MMP-specific activity assay (Biotrak, Amersham Bioscience, Freiburg, Germany) (Hanemaaijer et al., 1998). The range of the MMP-2 activity assay was 0.75-12.00 ng/ml with a sensitivity of 0.50 ng/ml. For MMP-9 the assay range was 2-32 ng/ml with a sensitivity of 1 ng/ml.

CD163 proteolytic activity measurements

CHO cells stably transfected with human full length CD163 (Kristiansen et al., 2001) were seeded in a 96 wells plate in a concentration of 200.000 cells per well. Cells were cultured for 48 hrs at 37°C in a CO_2 incubator with the addition of 20% plasma in protein free CHO cell medium (HyQ CCM5, Hyclone, Belgium). The experiments were performed in triplicate. MMP inhibitor (BB3103, British Biotech Pharmaceuticals Ltd (Oxford, UK) was used at a final concentration of 10 µM and vehicle controls (0.1% DMSO) were included. To asses the shedding-promoting activity of plasma we selected plasma samples based on low or high levels of sCD163 (mean sCD163 $\pm$ 1-2x standard deviation (SD)) assuming that sCD163 correlated with the shedding activity of the plasma. After 48 hrs cells were harvested and stained for CD163 and measured in the flow cytometer. In short, cells were stained with EDHu-1 (10 µg/ml) in PBS-0.1% BSA for 1 hr. Subsequently, cells were stained with FITC-conjugated-rabbit anti mouse (1:300, DAKO; Copenhagen, Denmark) for 45 min. After washing, cells were

resuspended in PBS-0.1% BSA and fluorescence intensity was determined using a FACS-calibur flow cytometer (Becton and Dickinson, San Jose, CA, USA). A minimum of 10,000 events was collected in list mode on FACS-calibur software.

Cytokine ELISA and cortisol measurement

IL-6, IL-10, and IL12-p40 in plasma were determined by ELISA (all from BioSource International Inc.). The lower detection limits of the cytokine ELISA ranged from 1 to 10 pg/ml.

Plasma cortisol was analyzed using solid-phase, chemiluminescent immunoassay (immunolite 2000, Diagnostic Products Corporation (DPC), Los Angeles, USA) with a detection limit of 5.5 nmol/l.

Statistical Analysis

Data of all parameters were tested by non-parametric tests (Mann-Whitney test, Kruskal Wallis test, and Spearman's correlation). A two-tailed p-value of less than 0.05 was considered to indicate statistical significance (confidence level 95%). Statistical analysis was performed using SPSS 11.0 (SPSS Inc., Chicago, IL, USA).

	HC	MS
Soluble CD163 (mg/l) [#]	1.8 ± 0.6	2.1 ± 0.6
Membrane CD163 (MFI)	56.1 ± 14.0[*]	42.7 ± 15.4
Cortisol	249.2 ± 76.0	352.2 ± 157.1[*]
MMP-2 (ng/ml)	429.9 ± 37.3	400.6 ± 45.4
MMP-9 (ng/ml)	10.8 ± 6.2	11.5 ± 7.2
Soluble MR	12.2 ± 3.3	13.9 ± 4.6
IL-6 (pg/ml)	172.7 ± 234.2	149.0 ± 387.2[*]
IL-12p40 (pg/ml)	93.2 ± 209.4	43.2 ± 105.1[*]
IL-10 (pg/ml)	94.2 ± 254.9	23.9 ± 86.7

Table 2: *Mean levels (± SD) of various markers measured in HC and MS patients*

[*] Values are considered significantly different (Kruskal Wallis test, p value < 0.05).
[#] Soluble CD163 was also measured in a cohort with 29 MS patients and 473 MS patients (mean MS patients: 2.2 ± 1.7 and HC: 1.5 ± 0.7).

Results

Shedding of CD163 is enhanced in Multiple Sclerosis

In order to investigate the possible regulation of shedding of CD163 in MS we measured sCD163 levels in the plasma of a large cohort (n=473) of MS patients and compared it to that in HC (n=29). A significantly enhanced level of sCD163 was observed in MS patients (Mann-Whitney U test, p value = 0.003) (Figure 1A). The higher sCD163 levels in this cohort of MS patients could mainly be attributed to the relapsing MS patients (Kruskal Wallis, p value = 0.046, data not shown). There was no significant difference between HC and progressive patients.

An additional group (34 MS patients and 10 HC) was subjected to further analyses to provide insight into the possible mechanisms of shedding and its relation to circulating cytokine levels. The mean of this group was representative of the larger cohort tested previously (Table 2). First, we evaluated the membrane expression of CD163 on all gated monocytic cells by flow cytometry. The average outcome measure (i.e. mean fluorescence intensity, MFI) of membrane CD163 appeared to be significantly lower (Mann-Whitney U test, p value = 0.02) in MS patients as compared to HC (Figure 1B, for means see Table 2). Within the HC group there was a positive correlation between membrane and sCD163 levels (Spearman's Rho = 0.711, p value = 0.002, Figure 1C). This correlation was lost in MS patients (Spearman's Rho = -0.183, p value = 0.427), consistent with a more complex regulation of CD163 metabolism in inflammatory conditions. Further analysis within the MS population showed that patients with a relatively long disease duration (14-35 years, n=6) have a significantly lower membrane CD163 expression compared to HC (Mann-Whitney U test, p value = 0.03). On the contrary, sCD163 levels showed a weak positive correlation with disease duration in MS patients (Spearman's Rho = 0.326, p value = 0.044). Collectively, these data are consistent with an increased, and perhaps progressive, proteolytic shedding of CD163 in MS patients.

In previous studies we established that glucocorticoids show a potent upregulation in CD163 membrane expression *in vitro* (Hogger et al., 1998; Van den Heuvel et al., 1999), as well as *in vivo* (Chapter 7). To investigate whether differences in CD163 levels were based on differences in endogenous cortisol levels we measured plasma cortisol levels in control subjects and MS patients. Cortisol levels were slightly higher in MS patients than in HC (for means see Table 2), however there was no correlation between membrane CD163 expression and cortisol levels in MS patients or HC, neither was there for sCD163 levels. This indicates that other disease specific or inflammation specific processes may be responsible for lower membrane expression of CD163 in MS patients.

Upregulation of a proteolytic CD163 shedding activity in Multiple Sclerosis

There is considerable evidence supporting CD163 shedding as a MMP-dependent process (Hintz et al., 2002; Matsushita et al., 2002; Timmermann and Hogger, 2005). However, the putative MMP's that are involved have remained elusive. We measured

plasma levels of MMP-9 and MMP-2, which are considered to be major plasma MMP's in MS (reviewed in (Yong et al., 2001)) and correlated these to sCD163 levels. MMP-2 and MMP-9 levels were not significantly different between HC and MS patients. A positive correlation of sCD163 with MMP-9 levels was detected in the in HC group (Spearman's Rho = 0.7, p value = 0.03; Figure 1D), but this was lost in MS patients (Spearman's Rho = -0.07, p value = 0.75). This is consistent with a putative role for MMP-9 in CD163 shedding, but also suggests that particularly in MS other proteinases may be more important.

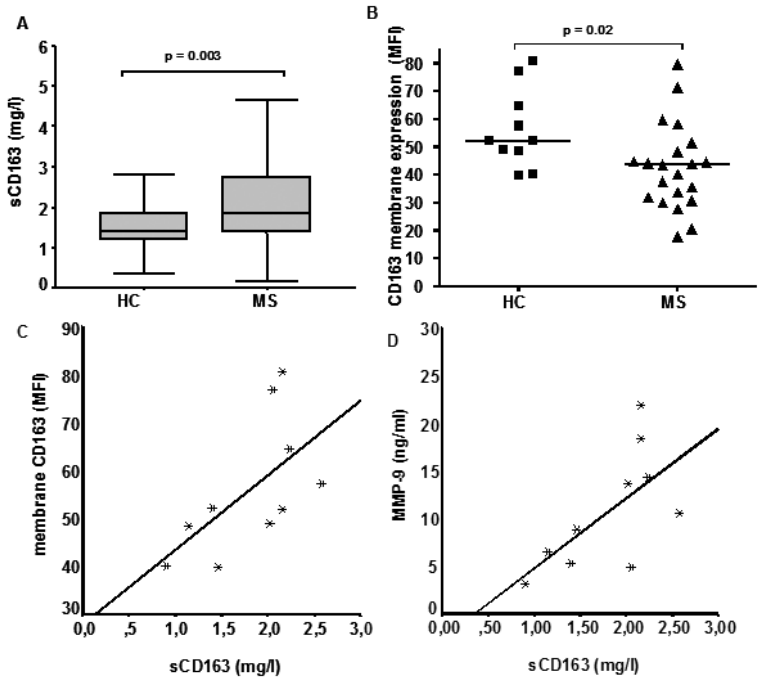

Figure 1: *CD163 shedding in Multiple Sclerosis*

A. sCD163 measured by sandwich ELISA in 473 MS patients and 29 HC. The mean of the MS patients (2.21 mg/l) is significantly higher (Mann-Whitney U test, p value = 0.004) compared to HC (1.60 mg/l). **B.** CD163 membrane expression on monocytes measured by flow cytometry staining of CD14 stained PBMC in 10 HC and 34 MS patients. The mean fluorescence intensity (MFI) reflects a combined effect of an increase in percentage of CD163 positive cells and the increase in CD163 expression per cell. CD163 membrane expression is significantly higher in MS patients compared to HC (Mann-Whitney U test, p value = 0.02). **C.** Scatter plot of sCD163 and membrane CD163 in HC. A positive correlation between sCD163 and membrane CD163 is observed, indicating that a high membrane CD163 expression results in high levels of sCD163 (Spearmans Rho: 0.71, p value = 0.002). In MS patients this correlation could not be observed. **D.** Scatter plot of sCD163 levels of 10 HC related to paired MMP-9 levels, an increase in sCD163 positively relates to an increased MMP-9 (Spearmans Rho: 0.70, p value = 0.03).

Since similar mechanisms for shedding have been postulated for the mannose receptor (MR), as for CD163, it was of interest to study whether sMR would correlate to MMP-2, MMP-9 or sCD163 levels. The MR is present on dendritic cells and macrophages and the receptor can be shed directly from the membrane resulting in a fully functional

soluble form of the molecule (Martinez-Pomares et al., 1998; Jordens et al., 1999). An important role for MMP's in the shedding of the MR has been postulated, similar to CD163. Therefore we suggested similar shedding mechanisms for both receptors, however, no correlation between sMR and sCD163, or sMR and MMP-2 or MMP-9 was observed (data not shown).

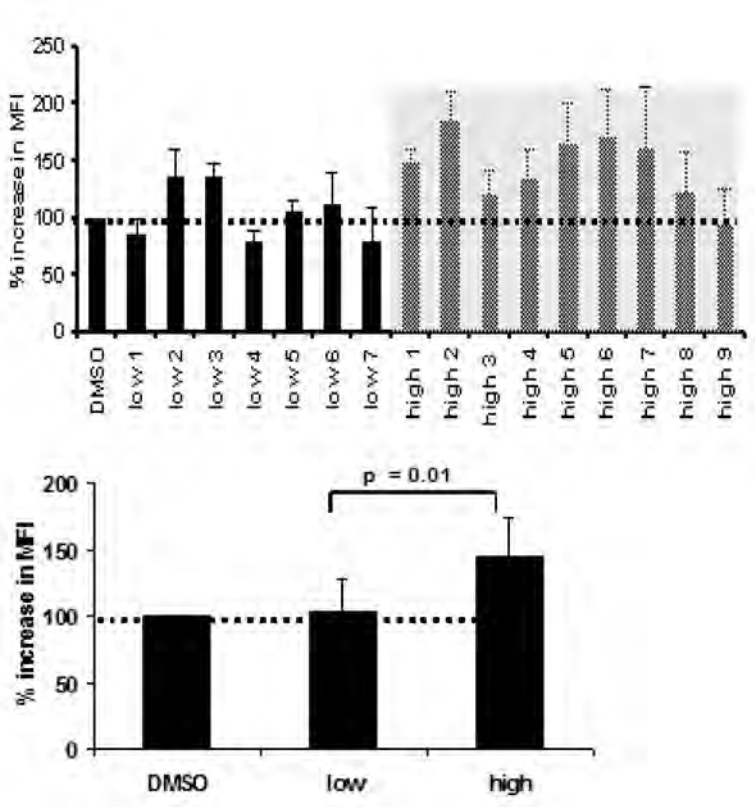

Figure 2: *Plasma induced shedding of CD163 inhibited by MMP inhibitor*

On the y-axis the percentage of plasma-induced shedding is shown calculated as MFI CD163 with inhibitor/MFI CD163 without inhibitor *100%. A value of 100% indicates that there is no effect of the MMP inhibitor and therefore no MMP dependent shedding has occurred in the samples. A percentage higher than 100% indicates that an increase in membrane CD163 has occurred when MMP inhibitor is added. In all MS patients with high sCD163 levels (mean + >1x SD) a decrease in CD163 membrane expression is seen, partially reverted by a broad spectrum MMP inhibitor (BB3103) in 8 out of 9 patients. Patients with low sCD163 levels (mean - >1x SD), showed variable results, 5 out of 7 patients showed no shedding after incubation with plasma, 2 HC unexpectedly showed an MMP dependent shedding. When all MS patients are taken together (bottom panel) this shows that a high sCD163 relates to MMP dependent shedding of membrane CD163 (as indicated by an increase of more than 100%). When the patients with low sCD163 levels are taken together this shows similar results as the vehicle control group (DMSO), indicative of no MMP-dependent shedding (levels remain at 100%, indicating that membrane CD163 expression remains stable) (Mann-Whitney U test, p value = 0.01).

In order to directly monitor the activity of MMP's in plasma of HC and MS patients for CD163 cleavage we measured the levels of membrane CD163 on CD163-transfected CHO cells by flow cytometry after incubation (for 2 days) with 20% plasma from MS patients. MMP-dependence was established by adding the broad spectrum MMP inhibitor (BB3103) or a vehicle control. A significant total plasma MMP activity, expressed as the ratio of membrane CD163 expression in the presence and absence of BB3103, was observed in 8 out of 9 MS patients with high plasma sCD163 levels, whereas little or no activity was found in MS patients with low plasma sCD163 (Figure 2). These results suggest that in the plasma from a subgroup of MS patients MMP's are present and are capable of proteolytically cleaving of membrane CD163, resulting in high sCD163 levels.

Shedding of CD163 is associated with decreased plasma pro-inflammatory cytokine levels

The cross-linking of CD163 on the surface of monocytes/macrophages by either Hb-Hp complexes (Philippidis et al., 2004) or antibodies (Van den Heuvel et al., 1999) triggers the production of inflammatory cytokines (e.g. IL-6 and IL-10). In order to investigate a possible relationship between membrane CD163 expression and cytokine production, the levels of IL-6, IL-10 and IL-12p40 were measured in the plasma of HC and MS patients. A significant decrease in IL-12p40 and IL-6 was observed (Table 2) within MS patients compared to the control group (Mann-Whitney U test, p value = 0.04 and 0.02 respectively). IL-10 levels, which varied considerably among individuals, were not significantly different between the two groups. Both IL-12p40 and IL-6 showed a positive correlation with membrane CD163 expression (Figures 3 A/B, Spearman's Rho = 0.49, p value = 0.005, and Spearman's Rho = 0.71, p value = 0.02), suggesting perhaps a direct link between plasma cytokine levels and functional CD163 expression on monocytes in MS.

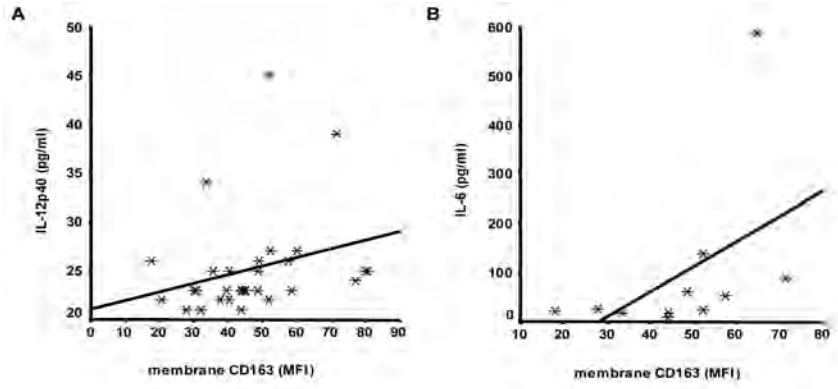

Figure 3:

A. IL12-p40 levels in plasma of HC and MS patients measured by ELISA and correlated to membrane CD163 expression (Spearman's Rho = 0.49, p value 0.005). **B.** IL-6 levels in plasma of HC and MS patients measured by ELISA and correlated to membrane CD163 expression (Spearman's Rho = 0.71, p value = 0.01).

Discussion

The human scavenger receptor CD163 is selectively expressed on mature tissue macrophages and monocytes. It has been implicated to play a role in both homeostatic and inflammatory processes. Elevated levels of sCD163 have been reported in several inflammatory conditions (e.g. RA, pneumonia, sepsis) (Moller et al., 2002a; Moller et al., 2004; Weiss and Schneider, 2006; Moller et al., 2006), and may have anti-inflammatory properties, for example via the inhibition of lymphocyte activation and proliferation (Hogger and Sorg, 2001; Frings et al., 2002; Baeten et al., 2004). To date CD163 has been studied in brain sections in MS (Chapter 4) (Boven et al., 2006), however, the systemic regulation of CD163 was unknown. Therefore, we studied sCD163 levels and monocyte CD163 membrane expression in MS patients. By means of ELISA we analyzed sCD163 in plasma samples of a group of MS patients and found a significant increase in sCD163 in MS. At the same time membrane expression of CD163 on monocytes isolated from MS patients showed a significant decrease in MS patients compared to HC. These findings are consistent with increased proteolytic shedding of CD163 during MS.

One possible explanation for the increased shedding of CD163 in MS that we explored is an elevated level of CD163-cleaving proteinases in plasma. sCD163 is a shedding product of membrane bound CD163 due to MMP-dependent cleavage (Timmermann and Hogger, 2005). An increase in proteinase activity maybe the reason for an increase in shedding of CD163. In MS an increase in MMP-9 mRNA expression has been described previously (Lichtinghagen et al., 1999). However, in MS patients we do not find a correlation between sCD163 and MMP-9 levels, whereas we did find such a correlation in HC. Apparently the regulation of shedding and expression of membrane CD163 is more complex in MS than it is in HC, therefore we cannot exclude that during inflammation other MMP's play a role.

Second, there might be another source of sCD163. In MS the main focus of inflammation lies in the CNS and previous reports by our group have shown an increase of CD163-positive macrophages in MS lesions (Chapter 4). sCD163 levels in cerebrospinal fluid (CSF) are higher than might be suggested from its molecular weight and hereby local production of sCD163 in the CNS can be concluded (preliminary unpublished data). Due to an overflow of sCD163 from the CSF the correlation between sCD163 and membrane CD163 expression in the periphery could be affected by local CD163 shedding.

sCD163 is associated with inflammatory conditions (Moller et al., 2002a) and membrane CD163 expression with anti-inflammatory alternative macrophage activity (Mantovani et al., 2002; Gordon, 2003). Therefore, we analyzed pro- and anti-inflammatory cytokine (IL-12p40, IL-6, and IL-10) and correlated their levels to sCD163 and membrane CD163 expression. We observed a decrease in IL-12p40 and IL-6 in MS correlating with membrane CD163 levels, but not sCD163 levels. It has been reported that CD163 triggering with the anti-CD163 antibody EDHu-1 leads to the production of IL-6 (Van den Heuvel et al., 1999).

Collectively we have provided evidence for an enhanced proteolytic shedding of CD163 in MS, which is associated with decreased plasma cytokine levels (IL-6 and IL-12p40).

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7.

Regulation of monocyte CD163 expression during glucocorticoid therapy: A prognostic marker for glucocorticoid treatment response?

Submitted

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Abstract

CD163 is a hemoglobin scavenger receptor expressed on tissue macrophages and a subpopulation of monocytes. Previous studies have shown that *in vitro* stimulation with the glucocorticoid dexamethasone, strongly upregulates CD163 expression on human blood monocytes. Glucocorticoid (GC) therapy is commonly used to treat (chronic) inflammatory diseases, such as Multiple Sclerosis (MS), but the treatment response differs among patients, suggesting differences in sensitivity.

We evaluated membrane monocyte CD163, soluble CD163 and CD163 mRNA in 34 MS patients during different time-points of GC therapy and tested whether *in vitro* response to GC is a good predictor for clinical response to the treatment.

A strong upregulation of monocyte CD163 expression was observed during GC therapy *in vivo*. However, measurement of sCD163 did not reveal significant differences during iv-MP therapy and CD163 mRNA showed, after correction for the induced lymphopenia by GC, only a small non-significant increase. Moreover, the *ex vivo* inducibility of membrane CD163 by GC on monocytes was significantly higher in clinical responders to GC as compared to non-responders to GC.

Expression of CD163 is regulated *in vivo* by GC in MS patients. Moreover, we identified CD163 as a possible prognostic marker for GC responsiveness.

Introduction

Glucocorticoids (GC) are commonly used as therapy in a variety of acute and chronic inflammatory conditions, including Multiple Sclerosis (MS), asthma, and rheumatoid arthritis (RA). GC are known to posses both immune-suppressive and anti-inflammatory effects, which they exert by affecting multiple cell types and activities (Charmandari et al., 2004). However, the physiological response and sensitivity to GC varies among species, individuals, tissues, cell types, and during the cell cycle (Bamberger et al., 1996). About one third of the patients with inflammatory disease respond poorly to GC treatment (Corrigan et al., 1991; Norman and Hearing, 2002; Kino et al., 2003). The underlying mechanism(s) responsible for these differences in individual GC responsiveness are essentially unknown and, perhaps even more important, prognostic tests for GC responsiveness are not available.

In MS, and in particular in the predominant relapsing-remitting (RR) form of the disease, GC are frequently administered at the time of an acute relapse, usually as a short course of high dose intravenous methylprednisolone (iv-MP) (Andersson and Goodkin, 1998). This results in an improved recovery of disability and a shortening of the relapse. In addition, MP treatment can be beneficial during the chronic progressive phase of MS (Andersson and Goodkin, 1998). Although the mode of action of GC in MS is not exactly known, it seems possible that GC exert their anti-inflammatory action, at least in part, by preventing disruption of the blood brain barrier, by decreasing leukocyte migration, and by inhibiting the production of pro-inflammatory mediators (Pozzilli et al., 2004).

Previous studies have shown that *in vitro* stimulation with the GC dexamethasone, strongly upregulates CD163 expression on human blood monocytes (Hogger et al., 1998; Van den Heuvel et al., 1999; Buechler et al., 2000; Sulahian et al., 2000; Pioli et al., 2004). CD163 is a member of the scavenger receptor cysteine-rich family group B that is exclusively expressed on cells of the macrophage lineage (Hogger et al., 1998; Van den Heuvel et al., 1999). It has been identified as a scavenger receptor for hemoglobin-haptoglobin complexes (Kristiansen et al., 2001) and ligation of CD163 triggers cytokine production (Van den Heuvel et al., 1999; Moestrup and Moller, 2004; Philippidis et al., 2004). A soluble form of the receptor has also been described and elevated levels of soluble CD163 (sCD163) have been reported in various inflammatory diseases (Moller et al., 2002a; Baeten et al., 2004; Moller et al., 2004; Hiraoka et al., 2005; Schaer et al., 2005). Furthermore, there are strong indications that CD163 plays an active role in the inflammatory response and pro- and anti- inflammatory mediators tightly regulate CD163 protein expression (Van den Heuvel et al., 1999).

In inflammatory lesions present in the brain of MS patients there appears to be an upregulation of CD163 expression on local macrophage subsets (Chapter 4). Furthermore, we have recently found that CD163 expression on monocytes is decreased in MS patients compared to healthy controls, which appears to be due to an increased level of CD163 shedding in MS (Chapter 6).

In the present study, we investigated whether CD163 expression on monocytes could be regulated during high-dose GC therapy in MS patients *in vivo*, and whether this is associated with the clinical response to GC.

Patients and methods

Thirty-four MS patients, scheduled to receive 1000 mg intravenous (iv)-MP for 3 consecutive days, were included in this study. The clinical characteristics of all patients are summarized in Table 1. The study was approved by the ethics committee of our hospital. Blood was collected on the first day of the treatment prior to the infusion of the first dose of iv-MP (baseline). After 2 days of treatment (i.e. after a cumulative dose of 2000 mg iv-MP) blood was collected before the final dose of iv-MP. Approximately 6 weeks after iv-MP treatment (post-MP) the clinical response of the treatment was evaluated and blood was collected.

	Relapsing MS	Progressive MS
Number	23	11
Male/Female	6/17	7/4
Age (years)	39.1 ± 9.7	52.7 ± 10.0
Disease duration (years)	8.2 ± 7.3	13.7 ± 8.0
EDSS	3.7 ± 1.7	5.1 ± 1.9
MP treatments (n)	4.7 ± 4.2	5.3 ± 5.2
Clinical Improvement:		
No	5	3
Minor	8	1
Significant	10	7

Table 1: *Patient characteristics*
EDSS: expanded disability status scale.

Peripheral blood mononuclear cell (PBMC) were isolated by Ficoll-IsoPaque density gradient centrifugation and membrane CD163 expression, sCD163 levels in plasma, and CD163 messenger RNA (mRNA) were studied by flow cytometry (described below), sandwich ELISA (performed as previously described) (Moller et al., 2002b), and quantitative polymerase chain reaction (qPCR; described below), respectively.

PBMC were cultured in RPMI1640 medium (Gibco Europe, Breda, The Netherlands) supplemented with 10% fetal calf serum and antibiotics, and cultured for 2 days in the presence or absence of 10^{-6} M Dexamethasone (DEX; Sigma, St Louis, USA) (only for baseline samples). The relative DEX-induced increase of CD163 membrane expression on monocytes and sCD163 levels in the supernatants was determined.

The effect of treatment was determined by the treating physician, who was masked for the laboratory results. Responders were defined as having functional improvement and/or a significant reduction of abnormal signs at neurological examination concerning the symptoms for which the MP treatment was given. The study was performed double-blinded and clinical data were disclosed after all laboratory tests had been performed.

Flow cytometry

Dual-color immunofluorescence was performed on thawed PBMC using anti-human CD163 (EDHu-1) (Van den Heuvel et al., 1999), followed by FITC-conjugated-rabbit anti mouse (DAKO; Copenhagen, Denmark). Subsequently, cells were pre-incubated with 2% mouse serum prior to staining with phycoerythrin (PE)-conjugated anti-human

CD14 (CD14-PE; BD Bioscience Pharmingen, San Diego, CA). Flow cytometric analysis of the cell fractions was performed as described in Figure 1. A minimum of 100.000 events was collected by FACScalibur (Becton Dickinson, Mountain View, CA).

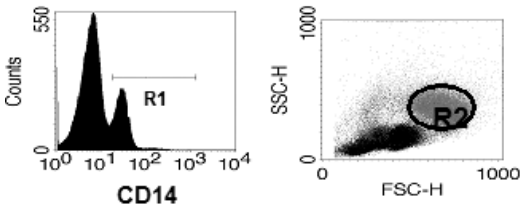

Figure 1: *Data analysis of monocyte CD163 expression by flow cytometry*

Flow cytometric data analysis of CD163 expression on peripheral blood monocytes was performed by using CD14 expression (**left**: selected in R1). The two-parameter correlated histogram (FSC vs. SSC, **right**) was then used to specifically set a gate around the monocytic cell population (shown in red, R2) and exclude cell debris and lymphocytes and granulocytes. The gated cells were replotted on histograms for FL1 (CD163) to calculate the percentage of positive cells and mean fluorescence (MFI) statistics (not shown). The percentage of CD14 cells within the monocyte gate (R2) was similar (80-90%) for all samples analyzed in this study.

Q-PCR

Total RNA was isolated from 5 million PBMC using the QIAamp® RNA blood mini kit from Qiagen (Albertslund, Denmark). In addition, total RNA was extracted from Flp-In CHO cells stably transfected with CD163 short tail variant as described (Nielsen et al., 2006) for use as calibrator material in qPCR. A standard curve was included in each run by inclusion of a series of dilutions of RNA from CD163 transfected cells. Real-time q-PCR was performed in a LightCycler® system (Roche Diagnostics, Hvidovre, Denmark) using forward primer 5'-ACA TAG ATC ATG CAT CTG TCA TTT G-3' and reverse primer 5'-CAT TCT CCT TGG AAT CTC ACT TCT A-3'.

Data were analyzed by non-parametric testing using SPSS 11.0 software (SPSS Inc., Chicago, IL, USA). A two-tailed p-value of less than 0.05 was considered to indicate statistical significance (confidence level 95%).

Results and discussion

Patient Characteristics

Of the 34 MS patients with relapsing MS and 11 patients with progressive MS, 29 patients had previously been exposed to systemic GC. The number of previous iv-MP treatments ranged from 0-19, and the minimum interval between the last exposure to GC and blood withdrawal was 30 days (for patient characteristics see Table 1).

Upregulation of CD163 by GC in vivo

CD163 is a scavenger receptor expressed on monocytes (Van den Heuvel et al., 1999; Sulahian et al., 2000) and can be upregulated after *in vitro* stimulation with GC (Hogger et al., 1998; Buechler et al., 2000; Van den Heuvel et al., 1999). To study whether CD163 expression could be regulated during GC therapy *in vivo*, we evaluated monocyte CD163 at different time-points during iv-MP therapy in MS patients.

A strong and significant upregulation of CD163 expression was observed during GC therapy *in vivo* in all patients (Figure 2A/B) in all patients after 2 days of MP treatment. This increase was reversible as CD163 expression returned to baseline 6 weeks after MP treatment. This clearly demonstrated that CD163 expression on monocytes could be upregulated by GC in human subjects *in vivo.*

Besides showing a clear upregulation in membrane receptor expression we attempted to provide insight into the possible mechanism of CD163 regulation. We evaluated CD163 mRNA in order to investigate whether the GC therapy was directly promoting CD163 gene expression. There was an increase in CD163 mRNA of total PBMC after 2 days of treatment (data not shown). However, when this was corrected for the relative increase in monocytes resulting from the lymphopenia previously reported after GC treatment in MS (Oosterhuis et al., 1984; Bloemena et al., 1990), only a relatively small and non-significant increase in CD163 mRNA remained (Figure 2C). Although, this does not necessarily exclude the possibility that the increase in CD163 protein expression on monocytes is primarily regulated at the mRNA level. In fact, a previous report on the regulation of CD163 by GC in isolated monocytes *in vitro* has indicated that mRNA for the majority of CD163 splice variants is transiently elevated upon GC exposure, peaking at ~8 hours after exposure, and that this precedes the rise in CD163 protein (Hogger et al., 1998). Thus, it seems possible that the rise in CD163 mRNA had occurred before sampling.

An alternative mechanism that could potentially contribute to the regulation of membrane CD163 on monocytes is the proteolytic cleavage and shedding of the molecule by e.g. matrix metalloproteinases (Hintz et al., 2002; Matsushita et al., 2002; Timmermann and Hogger, 2005). However, measurement of sCD163 did not reveal significant differences during iv-MP therapy (Figure 2D).

Overall, there was no difference in CD163 response (membrane expression, levels of sCD163, and CD163 mRNA expression) to GC treatment *in vivo* between relapsing and progressive MS, the height of the EDSS scores, and disease duration (summarized in Table 2).

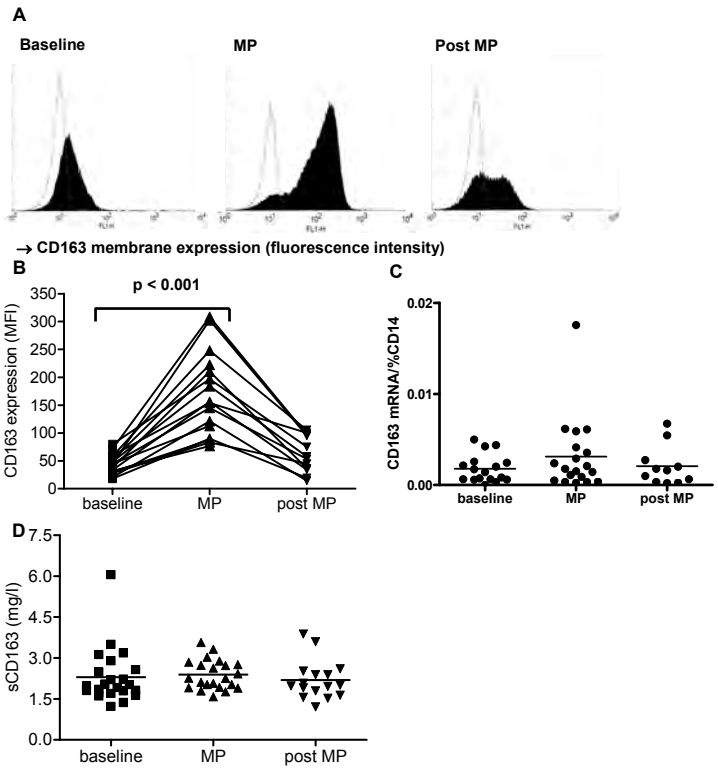

Figure 2: *CD163 levels after in vivo stimulation with GC in MS patients*

A. Typical example of CD163 membrane expression on monocytes (detected by EDHu-1 antibody) of an MS patient that received *in vivo* MP treatment. A transient increase in CD163 surface expression is seen both in the percentage of cells expressing CD163 as well as in the mean fluorescence intensity (MFI). **B.** Membrane CD163 expression (mean fluorescence intensity, MFI) of the gated monocytes (see Figure 1 for gating) at different time-points during iv-MP treatment. The time points sampled for each patient include baseline, after 2 days of treatment (i.e. after a cumulative dose of 2000 mg iv-MP; MP), and approximately 6 weeks after the iv-MP treatment (post-MP). Each line represents the response of one patient. During the MP-iv treatment there is a significant increase in membrane CD163 expression (Wilcoxon signed-rank test, $p < 0.001$). **C.** CD163 mRNA expression during MP treatment as analyzed by qPCR. CD163 mRNA has been divided through the percentage of CD14 positive cells in paired samples to correct for the induced lymphopenia occurring at day 2 of GC treatment (as can be seen in Supplementary Figure 2B). There is only a slight, albeit not significant, increase in CD163 mRNA per CD14 monocyte after 2 days of MP treatment. **D.** sCD163 levels in plasma during MP treatment measured by ELISA. No increase in sCD163 is observed during MP treatment.

CD163 as a prognostic parameter for GC responsiveness

We wondered whether there would be a difference for CD163 levels between responders and non-responders after *in vivo* GC treatment. For membrane CD163 and CD163 mRNA we observed no difference. On contrary, the relative increase of sCD163 upon treatment was significantly lower in responders as compared to non-responders (Mann-Whitney test, p value 0.04; Figure 3), but considering the small differences in absolute concentrations it seems rather unlikely that these amount to meaningful functional differences. Furthermore, there was no significant relation between age, MS subtype, disease duration or EDSS score and clinical response to GC treatment (summarized in Table 2). The apparent lack of relation between *in vitro* CD163 upregulation and clinical *(in vivo)* CD163 GC response was perhaps not surprising as CD163 regulation is presumably complex and systemic factors other than GC are likely to affect CD163 expression.

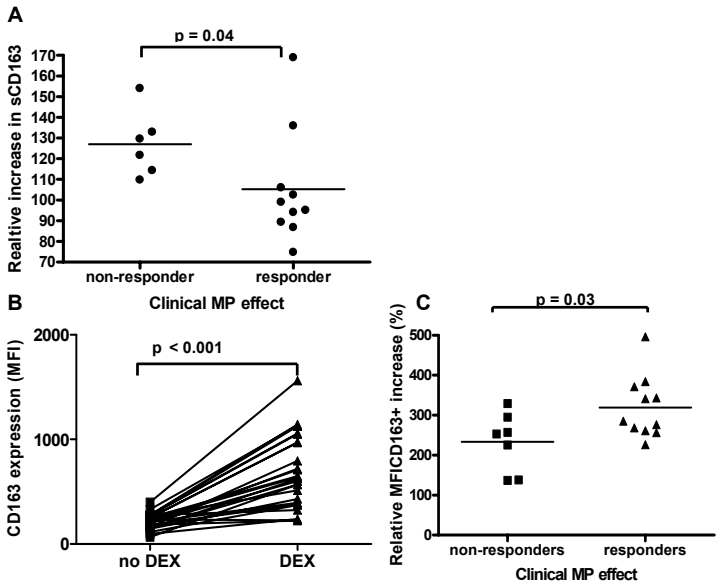

Figure 3: *Prognostic value for clinical response to GC after* ex vivo *CD163 inducibility with GC.*

A. Clinical responders to in vivo GC treatment show a significant lower relative increase in sCD163 levels during MP-iv treatment compared to non-responders of the treatment. The relative increase in sCD163 levels is defined as the ratio between sCD163 after treatment with MP-iv (day 2). sCD163 levels at baseline (day 0) * 100%. PBMC isolated from the blood of MS patients, taken before the start of iv-MP therapy, were cultured in the presence or absence of 10^{-6} M for 2 days and analyzed for monocyte CD163 expression. **B.** Line-graph of the CD163 membrane expression (MFI) on monocytes (see Figure 1 for gating). Each line represents CD163 expression in the PBMC fraction of 1 patient. **C.** Clinical responders to in vivo GC treatment show a significant lower relative increase in sCD163 levels during MP-iv treatment compared to non-responders of the treatment. The relative increase in sCD163 levels is defined as the ratio between sCD163 after treatment with MP-iv (day 2)/ sCD163 levels at baseline (day 0) * 100%.

	Relapsing/ progressive	EDSS	Disease Duration	Responders/non-responders
In vivo:				
MFI 163#	0.71	0.59	0.76	0.96
MFI 163+##	0.46	0.54	0.89	0.64
sCD163	0.75	0.17	0.57	**0.04***
CD163 mRNA	0.15	0.43	0.53	0.09
In vitro:				
MFI 163#	0.66	0.16	0.88	0.07
MFI 163+##	0.70	0.36	0.93	**0.03***
sCD163	0.19	0.38	0.49	0.29

Table 2: *P-values for comparisons between analysed parameters (indicated as relative increase from MP treatment to baseline value) and clinical parameters*

MFI CD163: CD163 membrane expression on gated monocytes, ## MFI CD163+: CD163 membrane expression on CD163 positive monocytes. CD163 expression, sCD163 and mRNA did not differ significantly between relapsing/progressive, EDSS, disease duration, and responders/non-responders on baseline (before MP treatment). Mann-Whitney statistical tests were used for comparison of relapsing/progressive and responders/non-responders with CD163 parameters. A p value <0.05 was considered significant (indicated by *). For EDSS-score and disease duration a Spearman's correlation was performed, values indicated are p-values, none of which were significantly different between the parameters tested.

The relative increase in CD163 expression is calculated from the ratio between CD163 membrane expression (MFI) on $CD163^+$ cells after culture with DEX / CD163 membrane expression on $CD163^+$ cells after culture without DEX * 100% in responders compared to non-responders of the *in vivo* MP-iv treatment. GC responders show a significant higher relative increase in CD163 membrane expression after DEX stimulation than non-responders (Mann Whitney test, p value 0.03).

However, it was of interest to establish a possible relation between GC responsiveness and CD163 inducibility by GC *ex vivo*. Therefore we collected blood at baseline from 24 (out of the 34) patients and cultured PBMC for 2 days with DEX and determined CD163 expression (Figure 3 A; Table 2). The relative DEX-induced increase in CD163 expression within the CD163-positive monocyte population was significantly higher in clinical responders compared to non-responders (Mann Whitney test, p value 0.03; Figure 3 B). Also, there was an inverse correlation between the relative increase of CD163 membrane expression and the relative decrease in sCD163 levels in the *in vitro* assays (Spearman's Rho: -0.51, p value = 0.01). This demonstrated that individual GC sensitivity was, at least in part, reflected in an intrinsic monocyte sensitivity towards GC, and that the relative increase in CD163 on monocytes *ex vivo* has prognostic value for the responsiveness of MS patients, but possibly also for those with other (chronic) inflammatory diseases, such as asthma or RA, that are frequently treated with GC.

Taken together, our findings show, for the first time, that a short-term treatment with a high dose of *in vivo* MP results in a transient increase in CD163 synthesis, similar to results described *in vitro.* Furthermore, our data suggest that CD163 has potential value as an *ex vivo* prognostic marker for GC responsiveness.

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8.

The rat macrophage scavenger receptor CD163 (ED2): expression, regulation and role in inflammatory mediator production

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Abstract

The monoclonal antibody ED2 is widely used to define macrophages in the rat. We have recently identified the ED2-antigen as the rat CD163 glycoprotein. CD163 is a member of the scavenger receptor cysteine-rich family group B (SRCR-B) and functions as a scavenger receptor for hemoglobin-haptoglobin complexes. Moreover, CD163 has also been indicated as a marker for alternatively activated macrophages. In the current study we identify rat CD163/ED2-antigen as a marker for mature tissue macrophages. Rat CD163 is constitutively expressed on most subpopulations of mature tissue macrophage, including splenic red pulp macrophages, thymic cortical macrophages, Kupffer cells in the liver, resident bone marrow macrophages, and central nervous system perivascular and meningeal macrophages, but is apparently absent from monocytes. Rat CD163 expression can be promoted by glucocorticoids, and this can be further enhanced by IL-4. Finally, engagement of rat CD163 on peritoneal macrophages induces the production of pro-inflammatory mediators, including nitric oxide, IL-6, and TNF-α. Collectively, our findings identify rat CD163 as a broadly expressed macrophage scavenger receptor that may play a role in the activation of macrophages during hemolytic and/or inflammatory conditions.

Introduction

Macrophages play important roles in normal and during various pathological conditions, such as (autoimmune) inflammation, infection, atherosclerosis and cancer. The major activities of macrophages, including phagocytosis and the production of inflammatory mediators, are controlled by surface receptors.

The surface glycoprotein CD163 is a member of the scavenger receptor cysteine-rich family group B (SRCR-B) and human CD163 has been shown function as a scavenger receptor for hemoglobin-haptoglobin complexes. This may contribute to the clearance of toxic free hemoglobin from the circulation and tissues (Law et al., 1993; Kristiansen et al., 2001). Engagement of human CD163 also results in the production of pro- and anti-inflammatory cytokines suggesting a potential role in host defense and/or inflammation (Van den Heuvel et al., 1999; Ritter et al., 2001; Philippidis et al., 2004).

Human CD163 is expressed on tissue macrophages and on a subset of monocytes (Van den Heuvel et al., 1999). Expression on monocytes can be upregulated by glucocorticoids (GC) and anti-inflammatory cytokines, such as IL-10 (Hogger et al., 1998; Van den Heuvel et al., 1999; Buechler et al., 2000). Consequently, CD163 has been indicated as a possible marker for so-called alternatively activated macrophages, which have been suggested to posses immune-suppressive activity (Gordon, 2003; Mosser, 2003) (Verreck et al., 2006). Thus far, little is known about the expression and function of CD163 in other species, such as rodents. We have recently demonstrated by purification and peptide sequencing that the rat ED2-antigen represents the rat ortholog of CD163 (Chapter 9). Our previous analysis has demonstrated that rat CD163/ED2 is expressed on subsets of tissue macrophages (Dijkstra et al., 1985; Barbé et al., 1990).

In the present study we extend these findings and confirm that rat CD163 is a marker for most mature tissue macrophages. Furthermore, we evaluate the regulation of rat CD163 by GC and IL-4. Finally, we provide evidence that rat CD163 upon ligation can trigger the production of pro-inflammatory mediators in macrophages.

Materials and methods

Animals

Male Wistar rats, between 7 and 12 weeks of age, were obtained from CPB-Harlan (Zeist, The Netherlands). Animals were kept under routine laboratory conditions and allowed free access to food and water. Microbiological status of the animals was according to FELASA recommendations.

Monoclonal antibodies

Monoclonal antibodies (mAbs) ED2 (IgG1, (Dijkstra et al., 1985) and BF5 (IgG1, against human CD4: a generous gift of Dr. J. Wijdenes, Diaclone Laboratories, Besançon, France) were purified from cell culture supernatants from hybridoma cells cultured in RPMI1640 containing 5% low IgG FCS (Life Technologies, Gaithersberg, MD) on protein A-Sepharose (Pharmacia, Uppsala, Sweden). Biotinylation was

performed using D-biotinoyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester (Boehringer Mannheim, Mannheim, Germany). To prepare endotoxin-free antibody (Ab) preparations, Abs were run over a polymyxin B column (Pierce, Rockford, IL), which when tested in the *Limulus* assay, yielded final LPS concentrations in culture of <5 pg/ml.

Immunohistochemistry for light microscopy

To study the ED2 mAb staining pattern in various organs and tissues, a two-step immunoperoxidase method was used. Cryostat sections (8 μm) were cut serially, picked up on gelatine coated slides and air-dried overnight in a container with silica gel. Immunohistochemistry was applied after 10 minutes fixation in dehydrated acetone. All wash steps were performed with 0.01M phosphate-buffered saline (PBS; pH 7.4) and the mAbs were diluted in PBS containing 1% bovine serum albumin (BSA; Organon Technika, Oss, The Netherlands). The ED2 antibody was used at optimal final dilution of 2 μg/ml. As a negative control the primary antibody was omitted. All incubations were carried out at RT. After incubation with the first antibody for 1 hr, the slides were rinsed and incubated with peroxidase conjugate. After 1 hr the slides were washed and incubated for 10 min with 3.3'-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, USA) in 0.05M Tris-HCl, (pH 7.6) containing 0.01% H_2O_2. After washing, sections were incubated for 20 min in 0.5% $CuSO_4$ solution and counterstained with haematoxylin, dehydrated and mounted in Entellan (Merck, Amsterdam, The Netherlands).

Cell populations and cell culture

The following cell lines were used: NR8383, a spontaneous alveolar macrophages tumour cell line (kindly provided by Dr. R. J. Helmke, University of Texas, San Antonio, TX (Helmke et al., 1987), and R2, a cell line of pleural macrophages induced by a silica injection in the pleural cavity of rats (a kind gift from D. Chao (Oxford, U.K.).
Primary alveolar macrophages were obtained by broncho-alveolar lavage with 5mM EDTA/PBS. Rat lymphocytes and thymocytes were isolated by fragmentation of the mesenteric lymph nodes and thymus, respectively, and mincing through a nylon gauze with RPMI1640 medium (Gibco BRL, Life Technologies Ltd. Palsley Scotland). Rat peripheral blood mononuclear cells (PBMC) were isolated from 5-7 ml blood by lymphoprep (density 1.077 g/ml) (Nyegaard & Co, Oslo, Norway). Peritoneal macrophages were harvested after peritoneal injection of RPMI1640 medium. Peritoneal cells (1.0 10^6/ml) were cultured for 3 days in RPMI1640 supplemented with 10% fetal calf serum (FCS) (Gibco), 2mM glutamine, 100U/ml penicillin and 100 μg/ml streptomycin. Spleen cell suspensions were enriched for macrophages by adherence to tissue culture plastic. The GC Dexamethasone (DEX) (10^{-6}M) (Sigma Chemical Co.St Louis, MO) and/or the rat recombinant cytokine IL-4 (5 U/ml) (McKnight et al., 1991) were added where indicated. For the *in vitro* ED2 functional assays cells were incubated overnight with 25 μg/ml purified endotoxin free ED2.

FACS analysis

Cell suspensions were washed in PBS containing 0.1% BSA and incubated with a saturating concentration (5 μg/ml) of monoclonal antibody ED2 or isotype control anti human CD4 (IgG1) diluted in PBS containing 0.1% BSA for 1 hr on ice. After washing, the appropriate conjugate (Streptavidin-Phycoerythrin (AVPE), Rabbit anti Mouse-Phycoerythrin (RAMPE) (Vector,laboratories, Burlingame, CA) supplemented with 1% normal rat serum was added and incubated for 30 min on ice in the dark. After several washing steps cells were resuspended in PBS containing 0.1% BSA and measured in a FACS-scan (Becton & Dickenson, Aalst, Belgium) and analysed using CellQuest software (Becton & Dickenson). Cells were gated, to exclude dead cells and other debris.

Analysis of nitric oxide production

The presence of nitric oxide (NO) in de peritoneal macrophage supernatants was determined by measuring the amount of nitrite, a metabolic product of NO. 100 μl of the culture supernatants were mixed with 100 μl Griess Reagent (0.1% naphtylene diamine dihydrochloride (Sigma), 1% sulfanulamide (Sigma) and 2.5% H_3PO_4 in distilled water). After 10 min incubation at RT the extinction of the reaction was measured using an ELISA reader (EL-312e, Bio-Tek Instruments) at 540 nm. The nitrite amount was calculated from a $NaNO_2$ standard curve. All incubations were performed in duplicate.

Cytokine measurements

IL-1β, IL-6 and TNF-α in cell culture supernatants were quantified by ELISA (NIBSC, Herfordshire U.K.). Microtiter plates (Nunc Maxisorp, Denmark) were coated overnight at 4°C with 100 μl coating antibody per well (final concentration 2μg/ml PBS). Plates were washed three times with washing buffer (0.5 M NaCl, 2.5 mM $NaH_2PO_4 \bullet H_2O$, 7.5 mM Na_2HPO_4, 0.1% Tween 20) and incubated overnight at 4°C with serial dilutions of the standard and cell culture supernatants. As a negative control, complete culture medium was used. All samples were tested in duplicate. After washing the plates, 100 μl detecting biotinylated antibody (diluted in wash/dilution buffer + 0.5% normal sheep serum) was added and the plates were left at RT for 1 hr. Plates were washed again and incubated with the conjugate Streptavidin/poly-HRP for 1 hr at RT.

Subsequently, plates were washed thoroughly, and 3,3',5,5'-tetramethylbenzidine (TMB; Sigma, St. Louis, USA) was used as a substrate. The reaction was stopped by addition of 1M H_2SO_4. The absorption was measured at 450 nm (Spectra Max, Molecular Devices). The amounts of IL-1β, IL-6 or TNF-α in the supernatants were determined using a standard curve.

Spleen	
White pulp	
PALS mϕ	+
Tingible body mϕ (TBM)	-
Marginal zone	
Marg. Metallophilic mϕ	-
Marg. Zone mϕ	-
Red pulp mϕ	+
Peyer's patches	
Interfollicular area mϕ	+
TBM	-
Villi mϕ	+
Lymph node	
Cortex	
Paracortical mϕ	+
TBM	-
Medulla mϕ	+
Subapsular mϕ	+
Subcapsular sinus mϕ	-
Thymus	
Medullary mϕ	-
Cortical mϕ	+
Subcapsular mϕ	+
Interlobular trabeculae mϕ	+
Liver	
Kupffer cells periportal	+
Kupffer cells pericentral	+/-
Lung	
Perivascular/Peribronch. mϕ	+
Alveolar mϕ	-
Pancreas	
Islets of Langerhans	-
Interlob. connect. tissue mϕ	+

Bone marrow	
Resident bone marrow mϕ	+
Kidney	
Mesangial mϕ	+
Mammary gland (lactating)	
Connective tissue mϕ	+
Ovaria	
Medullary mϕ	+
Primary follicle	-
Growing follicle	-
Mature Graafian follicle	-
Corpus luteum mϕ	+
Heart	
Myocardial mϕ	+
Endocardial mϕ	+
Stomach	
Mucosa	
Epithelial mϕ	-
Lamina propria mϕ	+
Muscularis mucosa mϕ	+/-
Submucosal mϕ	+
Muscularis externa mϕ	+
Colon	
Mucosa	
Epithelial mϕ	-
Lamina propria mϕ	+
Muscularis mucosa mϕ	+
Submucosal mϕ	+
Muscularis externa mϕ	+/-
Central Nervous System	
Perivascular mϕ	+
Meningeal mϕ	+
Microglial cells	-

***Table 1**: Distribution of CD163 in rat tissues analyzed by immunohistochemistry with ED2*
Positive (+) staining with the ED2 mAb; (+/-) very few cells stained; (-) no staining.

Results

Rat CD163 is selectively expressed by mature tissue macrophages

Previous studies have shown that ED2 specifically recognizes a subset of mature tissue macrophages in the rat, but not bone marrow macrophage progenitors, monocytes, granulocytes or dendritic cells (Dijkstra et al., 1985; Dijkstra and Damoiseaux, 1993; Damoiseaux et al., 1993). In this study we extend the tissue distribution analysis of the CD163 antigen by analyzing additional tissues and isolated cell populations (Figure 1 and Table 1). The main conclusion is that rat CD163 is exclusively expressed by most subpopulations of mature tissue macrophages. FACS analysis using the monoclonal antibody ED2 on isolated cells revealed that subpopulations of peritoneal macrophages (47%) and spleen macrophages are CD163-positive. All other cell types tested, including alveolar macrophages, monocytes, NR8383, R2, thymocytes and lymphocytes were apparently ED2-negative (Figure 1 and data not shown).

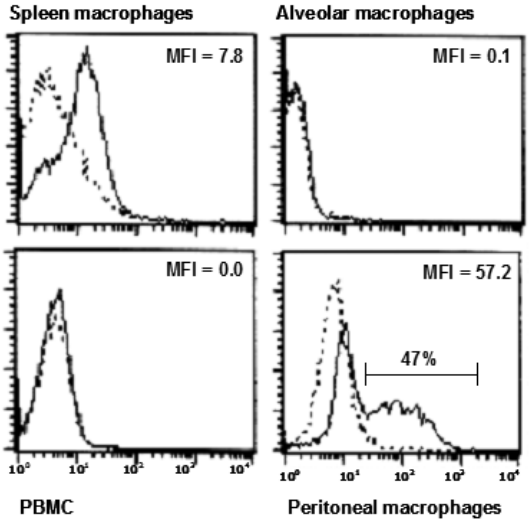

Figure 1: *Rat CD163 surface expression on freshly isolated macrophage populations analyzed by flow cytometry using ED2.*

The dotted line represents the isotype control and the solid line represents ED2 staining. MFI indicates the difference in fluorescence intensity between ED2 staining and conjugate control. Parallel stainings with the rat monocyte/macrophage marker ED1 indicate that 10-15% of the PBMC are monocytes (not shown).

Regulation of rat CD163 expression

It is known that the expression of human CD163 on monocytes can be promoted by GC and anti-inflammatory cytokines (Hogger et al., 1998; Van den Heuvel et al., 1999). Based on this, human CD163 has been suggested as a marker of alternatively activated macrophages (Gordon, 2003; Mosser, 2003), in spite of some conflicting data concerning IL-4 regulation (Van den Heuvel et al., 1999) (Buechler et al., 2000; Schaer

et al., 2002). It was therefore of interest to investigate the regulation of rat CD163 expression on macrophages by GC and cytokines or combinations of these. Peritoneal macrophages were cultured for three days in the presence or absence of these factors (Figure 2). In the absence of stimuli CD163 expression on peritoneal macrophages has virtually vanished after three days. However, in the presence of the GC DEX (10^{-6}M) CD163 expression was promoted on a subpopulation of peritoneal macrophages as compared to cells cultured in medium alone. Under influence of both DEX and IL-4, a similar subpopulation remains positive, albeit with higher CD163 expression. IL-4 alone did not influence CD163 expression. It has remained unclear whether the differences are the result of true upregulation, the lack of downregulation (i.e. maintenance), or a combination of these. When isolated rat monocytes or the rat macrophages cell lines NR8383 or R2, neither of which express detectable levels of CD163, were cultured in the presence of the above mentioned stimuli no detectable upregulation of CD163 could be observed (data not shown). Other stimuli like retinoic acid, lipopolysaccharide (LPS), phorbol esther (PMA), or other cytokines, IFN-β and IFN-γ, did not alter the expression of CD163 on peritoneal macrophages neither in the absence nor in the presence of DEX (res*ults not shown).*

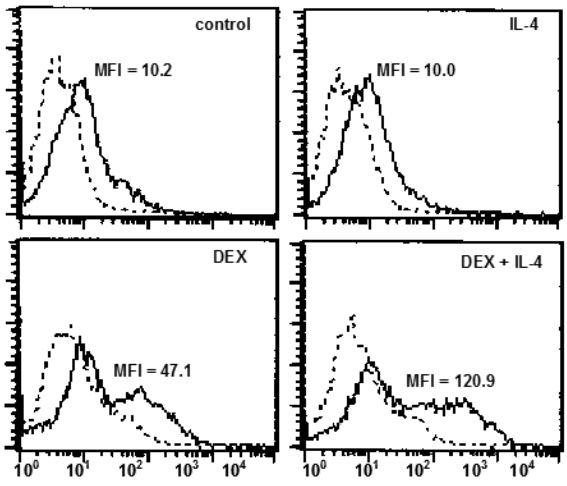

Figure 2: *Flow cytometric analysis of CD163 expression on rat peritoneal macrophages*

Peritoneal macrophages were treated with DEX (10^{-6}M), IL-4 (5 U/ml) or the combination of both, for three days. Dotted line represents the conjugate control and solid line the ED2 staining.

Engagement of rat CD163 triggers inflammatory mediator production

Previous studies have shown that cross-linking of human CD163 on GC-stimulated monocytes with either antibodies or hemoglobin-haptoglobin complexes induces the production of inflammatory cytokines (Van den Heuvel et al., 1999; Ritter et al., 2001; Philippidis et al., 2004). A possible role for rat CD163 in the production of inflammatory mediators by freshly isolated resting peritoneal macrophages was investigated upon CD163 engagement with the ED2 monoclonal antibody. This resulted in the secretion of NO, IL-1β, IL-6 and TNF-α (Figure 3). The NO response, which is described here for the first time, was comparable to the response evoked by 100ng/ml LPS. The secretion of IL-1, IL-6 and TNF-α was even higher. Isotype-matched control antibody did not induce a significant response. This shows that engagement of CD163 on rat macrophages is able to induce a potent pro-inflammatory mediator response.

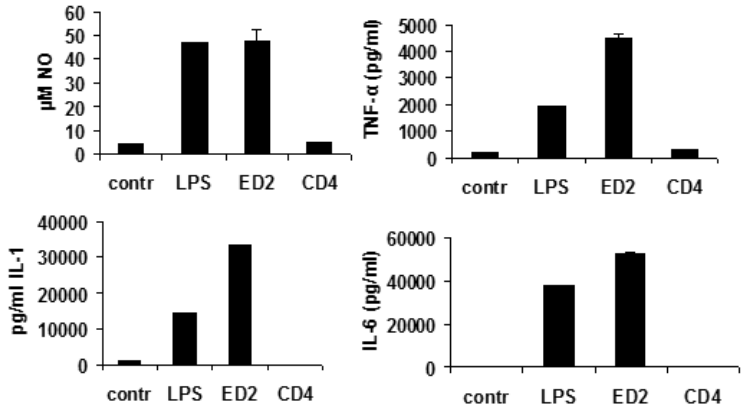

Figure 3: *Inflammatory mediator production upon CD163 engagement on rat peritoneal macrophages*

Peritoneal macrophages were cultured overnight in the presence of 25 µg/ml purified ED2 or isotype-matched control antibody BF5 (directed against human CD4). The production of NO, TNF-α, IL-1β, and IL-6 was measured in supernatants as described in materials and methods section. Results shown are means ± SD of duplicate incubations from a representative experiment.

Discussion

The ED2 monoclonal antibody is widely used as a marker for rat macrophages, but the nature of the surface glycoprotein has remained elusive. Recently, we have established the identity of ED2 antigen by micro-sequencing and demonstrated that it is the rat ortholog of CD163 (Chapter 9). The human and mouse CD163 molecules had previously been cloned and sequenced (Law et al., 1993; Schaer et al., 2001). The extracellular region of CD163 is composed of nine SRCR domains, and CD163 together with M160, CD5, CD6, WC1, Spα, Pema-SPERG, Ebnerin, CRP-ductin, Hensin, DMBT1, and gp-340 form group B of the SRCR family (Jones et al., 1986; Wijngaard et al., 1992; Mayer and Tichy, 1995; Li and Snyder, 1995; Cheng et al., 1996; Takito et al., 1996; Aruffo et al., 1997; Gebe et al., 1997; Mollenhauer et al., 1997; Holmskov et al., 1999). CD163 has been identified as an endocytic receptor for hemoglobin-haptoglobin complexes and is presumed to play a role in the clearance of the toxic free hemoglobin from tissues and/or the circulation (Kristiansen et al., 2001; Madsen et al., 2004; Nielsen et al., 2006). We and others have previously shown that engagement of CD163 on GC-stimulated human monocytes, either by antibodies or by its natural ligand, triggers the production of inflammatory cytokines, such as IL-1β, IL-6, GM-CSF and IL-10 (Van den Heuvel et al., 1999; Ritter et al., 2001; Philippidis et al., 2004). Evidence suggests that somehow casein kinase II (CKII), which specifically binds to the cytoplasmic domain of CD163, and protein kinase C are involved CD163 signalling (Van den Heuvel et al., 1999; Ritter et al., 2001). Our present findings suggest that the capacity to trigger inflammatory mediators is shared by rat CD163, which upon ligation triggers NO, TNF-α, IL-1β and IL-6 production by macrophages. Our studies were performed with freshly isolated peritoneal macrophages, of which only a fraction is $CD163^+$. However, the magnitude of the response suggests that the signal provided by CD163 with respect to inflammatory mediator production can be quite strong.

Our present findings, which complements our earlier studies (Dijkstra et al., 1985; Damoiseaux et al., 1993), demonstrate that rat CD163 is selectively expressed on mature tissue macrophages, and is apparently absent from monocytes and alveolar macrophages. While most subpopulations of rat mature tissue macrophages express CD163, there are some notable exceptions. These include e.g. the splenic marginal zone macrophages and marginal metallophilic macrophages, and microglia in the central nervous system. Expression of the human CD163, although not studied in as much detail as rat CD163, is in general agreement with this (Van den Heuvel et al., 1999). However, there are a few notable differences between rat and human CD163 in terms of expression pattern and regulation. While rats apparently lack CD163 expression on monocytes and alveolar macrophages (Figure 2), the molecule is reportedly expressed on these subsets in man (Zwadlo-Klarwasser et al., 1990; Van den Heuvel et al., 1999; Buechler et al., 2000). This difference in basal monocyte CD163 expression levels between rat and man can perhaps explain the apparent difference in CD163 regulation on tissue macrophages between these species. It seems possible that GC and IL-4 act essentially as enhancers of gene expression rather than being actual inducers. In any case, GC-mediated regulation of CD163 may be relevant

during inflammation. For example, the early upregulation of CD163 on resident brain macrophages (i.e. perivascular and meningeal macrophages) during rat experimental allergic encephalomyelitis (EAE) (Polfliet et al., 2002), follows a rise in systemic GC (Huitinga et al., 2000). Furthermore, we have recently shown that CD163 is upregulated in monocytes during GC therapy in Multiple Sclerosis patients (Chapter 6).

CD163 is frequently indicated as a marker for so called alternatively activated macrophages, but this designation can be misleading. While CD163 expression may distinguish between either GC/IL-4- or IFN-γ- treated human monocytes, it may not be an *activation* marker *per se*, unless most subpopulations of macrophages in the body are in a permanently alternatively activated state. In fact, our present functional experiments argue in favor of a possible causal link between CD163 and a pro-inflammatory (i.e. classical?) macrophages phenotype.

Collectively, it appears that the majority of mature tissue macrophages have a potent system, based on CD163 expression and signaling, for turning on pro-inflammatory mediator production. The major challenge will be to establish a possible relevance of this CD163 pathway during hemolytic and/or inflammatory conditions.

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9.

The macrophage CD163 surface glycoprotein is an erythroblast adhesion receptor

Blood in press

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Abstract

Erythropoiesis occurs in erythroblastic islands where developing erythroblasts closely interact with macrophages. The adhesion molecules that govern macrophage-erythroblast contact have only been partially defined. Our previous work has implicated the rat ED2 antigen, which is highly expressed on the surface of macrophages in erythroblastic islands, is involved in erythroblast binding. In particular, the monoclonal antibody ED2 was found to inhibit erythroblast binding to bone marrow macrophages. Here, we identify the ED2 antigen as the rat CD163 surface glycoprotein, a member of the group B scavenger receptor cysteine-rich (SRCR) family that has previously been shown to function as a receptor for hemoglobin-haptoglobin (Hb-Hp) complexes and is believed to contribute to the clearance of free hemoglobin. CD163 transfectants and recombinant protein containing the extracellular domain of CD163 supported the adhesion of erythroblastic cells. Furthermore, we identified a 13 amino acid motif (CD163p2) corresponding to a putative interaction site within the second SRCR domain of CD163 that could mediate erythroblast binding. Finally, CD163p2 promoted erythroid expansion *in vitro*, suggesting that it enhanced erythroid proliferation and/or survival, but did not affect differentiation. These findings identify CD163 on macrophages as an adhesion receptor for erythroblasts in erythroblastic islands and suggest a regulatory role for CD163 during erythropoiesis.

Introduction

The functional unit for definitive erythropoiesis in the bone marrow is the erythroblastic island, a multicellular structure composed of a central macrophage surrounded by erythroblasts at various stages of differentiation (Bernard, 1991; Hanspal, 1997; Chasis, 2006). The contact between erythroblasts and macrophages supports the growth, survival and differentiation of erythroblasts, and allows for phagocytosis of the extruded erythroid nucleus. Thus far, the molecular interaction(s) that mediate the formation of erythroblastic islands have only been partly defined (Morris et al., 1991; Chasis, 2006). Firstly, interactions between VCAM-1 on macrophages and α_4-integrins on erythroblasts have been implicated in the contact between these cells (Sadahira et al., 1995). Furthermore, the erythroblast macrophage protein (Emp) has been identified, which is expressed on macrophages, erythroblasts and other cells (Hanspal and Hanspal, 1994; Hanspal et al., 1998; Soni et al., 2006). Emp is believed to mediate erythroblast adhesion to macrophages probably by homophilic interaction. Of relevance, Emp-deficient foetuses, which die perinatally, have significantly reduced numbers of erythroblastic islands and defective erythropoiesis. This phenotype appears to result from a deficiency in both macrophage development as well as disturbed erythroblast nuclear extrusion. Finally, the erythroid intercellular adhesion molecule-4 (ICAM-4) has been demonstrated to bind to the α_5-integrin expressed by macrophages in erythroblastic islands and this has also been shown to contribute to erythroblastic island formation *in vivo* (Mankelow et al., 2004; Lee et al., 2006).

We have previously identified the rat macrophage ED2 antigen, which is expressed on resident bone marrow macrophages as well as other subsets of mature tissue macrophages, as a candidate receptor for erythroblasts (Barbe et al., 1996). In particular, we have shown that binding of rat fetal liver erythroblasts to resident bone marrow macrophages can be blocked by the ED2 monoclonal antibody (mAb). In order to provide further insight into the molecular interactions between erythroblasts and macrophages and the contributions of these interactions to erythropoiesis, we further characterized the role of the ED2 antigen in erythroblast-macrophage adhesion. Our results identify the ED2 antigen as the macrophage hemoglobin scavenger receptor cysteine-rich (SRCR) family member CD163. Furthermore, we show that CD163 can directly support interactions with erythroblasts and identify a motif in the second SRCR domain of CD163 that, by binding to a putative counter-receptor on erythroblasts, promotes their growth and/or survival. This implicates CD163 in erythroblast adhesion to macrophages and suggests a possible regulatory role for this molecule in erythropoiesis.

Materials and methods

FACS staining

Immunofluorescence staining of stably transfected CHO cells (Kristiansen et al., 2001) was performed on 100,000 cells using anti-human CD163 mAb (EDHu-1 (Van den Heuvel et al., 1999)). Cells were stained with EDHu-1 (10 µg/ml) for 1 hr at 4°C in PBS-0.1% bovine serum albumin (BSA; Boehringer-Mannheim, Mannheim, Germany), and washed. Next, the cells were stained with FITC-conjugated-rabbit anti mouse (1:300; DAKO; Copenhagen, Denmark) for 45 min. After washing, cells were resuspended in PBS-0.1% BSA and fluorescence intensity was determined using a FACS-calibur flow cytometer (Becton and Dickinson, San Jose, CA, USA).

Immunohistochemistry

Fresh spleen cryosections of 7 day old normal male DA rats and adult Lewis rats that had been infected with *Plasmodium berghei* 6 days before were double-immunostained for red pulp macrophages (ED2, peroxidase-black) and rat transferrin receptor (OX26, alkaline phosphatase-red) by the indirect immunoenzyme method as described previously (Matsuno K, 2001).

Affinity purification and peptide sequencing of the ED2 antigen

The mouse anti-rat mAb ED2 was described previously (Dijkstra et al., 1985; Barbe et al., 1990; Barbe et al., 1996) and is available from Serotec, Oxford, UK. The ED2 antigen was affinity isolated from a rat spleen lysate (i.e. 60 spleens from Wistar, Fisher F_1 and Fisher F_{334} rats homogenized in 1% NP40; 0.01 M Triethanolamine/HCl, pH 7.8; 0.15 M NaCl containing the protease inhibitors aprotinine, pepstatine, leupeptine and PMSF) on an ED2-CNBr-sepharose column (4.3 mg IgG/ml) after preclearance on a bovine IgG (10 mg IgG/ml) column. The ED2 antigen was eluted using 50mM Glycine, pH 2.5; 0.1% Triton X-100; 0.15 M NaCl yielding a total of ~100-120 µg protein. After further purification of the 175-kDa band (Figure 2A) on tricine SDS-PAGE and semi-dry blotting to PVDF membrane N-terminal sequencing was performed by Edman degradation on a protein sequencer (Applied Biosystems 473A).

Internal peptide sequences were obtained by two methods. Firstly, peptides from an endolysine C digest were separated by HPLC and sequenced as above. Secondly, ED2 antigen purified from a silver-stained acrylamide gel was subjected to Q-tof analysis (Micromass, Q-TOF). Where indicated fractions were treated with PNGase F (Biolabs, New England, USA) according to the manufacturer's instructions.

Isolation of erythroblasts

Rat foetal liver erythroblasts were isolated as previously described (Barbe et al., 1996). Human erythroblasts were generated *in vitro* from CD34-positive human stem cells, obtained from healthy donor umbilical cord blood, as previously described (Giarratana et al., 2005) with minor modification. Briefly, mononuclear cells were isolated from

umbilical cord blood by density centrifugation over a ficoll-paque solution. Magnetic activated cell sorting (MACS; Miltenyi Biotech, Auburn, CA) using a hapten-conjugated antibody against CD34 coupled to beads was used to isolate CD34-positive cells. CD34-positive cells were cultured at 10,000 cells/ml for 8 days in serum free medium containing the indicated sources or iron, transferrin and other supplements as well as stem cell factor, IL-3, EPO and hydrocortisone. After culture the purity (>95%) and maturity of erythroblasts was checked with May-Grunwald-Giemsa stainings of cytospin preparations. Both rat and human erythroblast suspensions were <1.5% positive for CD163 as evaluated by flow cytometry.

Transfectants and adhesion assays

K562 cells were labelled with 0.5 μM 2',7'-bis-(2-carboxyethyl) -5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) for 15 min in HEPES-buffered RPMI1640 (i.e. medium) at 37^{0}C, washed, and 100,000 cells in 100 μl medium were allowed to adhere for 1 hr at 37^{0}C to CHO cells stably expressing full-length human CD163 (Kristiansen et al., 2001) or to parental cells cultured to confluence in flat bottomed 96 well plates. After washing cells were lysed in 0.1 M NaOH and fluorescence was determined in a microplate reader (Fluostar Galaxy, BMG Laboratories, Offenburg, Germany) at an excitation of 485 nm and an emission of 535 nm. The % of adherence was calculated using a K562 cell calibration curve.

Solid phase binding of K562 cells or freshly isolated rat fetal liver erythroblasts (Barbe et al., 1996), using 100,000 cells/well (100 μl), to synthetic peptides was performed as described previously (Bikker et al., 2002; Bikker et al., 2004) omitting Tween-20 from the buffers. Adhesion of nucleated cells was quantified by SYTO-13 staining (Invitrogen) and measured in the microplate reader at 488 nm excitation and 509 nm emission.

In vitro erythroid maturation was performed by culturing rat erythroblasts in serum free medium (HyQ CCM5, Hyclone, Belgium) and 2 IU/ml recombinant human erythropoietin (EPO, Roche, UK) in triplicate. Cultured erythroblasts were detached and then counted using a hemocytometer. Maturation was determined by May-Grunwald-Giemsa staining of cytospin preparations.

Fluorescent bead adhesion assay

CHO cells were stably transfected with a plasmid to express the extracellular domain of CD163 (amino acids 1-993) as a fusion protein with human IgG1-Fc (generously provided by dr. R.J.J. van Neerven). Supernatants from these cultures were collected and CD163-Fc derived from stably transfected CD163 CHO cells was purified before coating onto carboxylate-modified TransFluoSpheres (488/645 nm, 1.0 μm, Molecular Probes, Eugene, OR) as described previously (Geijtenbeek et al., 1999). Briefly, streptavidin was covalently coupled onto TransFluoSpheres as described by manufacturer. To enable coupling of CD163-Fc, streptavidin-coated beads were allowed to bind to biotinylated goat-anti human anti-Fc Fab_2 fragments (6 μg/ml, Jackson ImmunoResearch Lab. Inc.) in 0.5 ml TBS containing 2 mM Ca^{2+} for 2 hr at 37°C. The beads were washed once with TBS-Ca^{2+} and incubated with human CD163-Fc (1 μg) for

2 days at 4°C. The ligand-coated beads were washed, resuspended in 100 µL TBS-Ca^{2+}, and stored at 4°C. For bead adhesion to K562 cells and rat erythroblasts, cells were resuspended in TBS-Ca^{2+}. The ligand-coated beads (20 beads/cell) were added to 100,000 cells and the suspension was incubated for 1 hr at 37°C. The percentage of K562 cells or rat erythroblasts binding CD163-coated beads was quantified by flow cytometry using the FACS-calibur (Becton Dickinson & Co, Oxnard, CA) and expressed as normalized values for comparison of individual experiments. As a control beads coated with human ICAM-3-Fc (van et al., 1996) were used.

Results

Expression of the ED2 antigen in erythroblastic islands

Our previous studies have provided preliminary evidence that the rat ED2 antigen is involved in the adhesion of erythroblasts to resident bone marrow macrophages (Barbe et al., 1996). In particular, the mAb ED2 was shown to inhibit erythroblast binding to resident bone marrow macrophages in bone marrow frozen sections (~80% inhibition), or freshly isolated resident bone marrow macrophages (~60% inhibition), respectively. Also, the ED2 antigen was found highly expressed on these resident bone marrow macrophages that form the central macrophages in erythroblastic islands in the bone marrow. Here, we further characterize the role of the ED2 antigen in erythroblast adhesion.

First, we investigated whether the ED2 antigen is expressed by macrophages in erythroblastic islands at extramedullary sites, such as the spleen. In particular we investigated the erythropoietic splenic tissue of both juvenile (one week old) rats and adult animals that had been infected 6 days before with the malaria parasite *Plasmodium brucei*. Typical erythroblastic islands, identified by staining with transferrin receptor/CD71 (using mAb OX26), containing macrophages expressing high levels of ED2 antigen were identified in the splenic red pulp area in both conditions (Figure 1). In fact, most if not all erythroblastic islands contained one or more ED2-positive macrophages. In contrary, the ED2-negative ED3-positive macrophages, which are located in the marginal zone (Van den Berg et al., 2001), did not form erythroblastic islands (data not shown). In normal adult spleens, which have considerably less erythropoietic activity, the ED2 antigen was still highly expressed on the red pulp macrophages (data not shown, also see (Dijkstra et al., 1985)), suggesting that the presence of the ED2 antigen on these macrophages *per se* is not sufficient for the formation of erythroblastic islands. Taken together, these findings demonstrate that the ED2 antigen is expressed on macrophages in erythroblastic islands at both medullary and extramedullary sites.

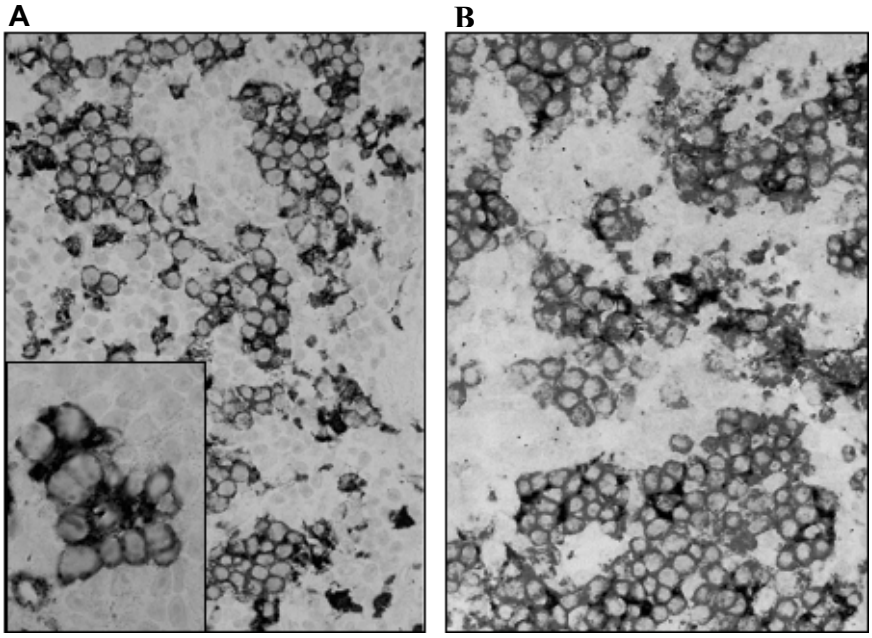

Figure 1: *Expression of the rat ED2 antigen by macrophages in splenic erythroblastic islands.*
For a full colour picture see Appendix. Microphotographs from the red pulp areas from the spleens of a juvenile 7 day old rat (**A**), or an adult animal 6 days after *Plasmodium berghei* infection (**B**), double stained with mAb ED2 for rat CD163 (black) and mAb OX2617 for rat transferrin receptor-positive erythroblasts (red).

The ED2 antigen is identical to the scavenger receptor CD163

In order to establish the molecular identity of the rat macrophage ED2 glycoprotein antigen we subjected a rat spleen lysate to affinity chromatography using a column containing the ED2 mAb coupled to sepharose. Previous work had shown that ED2 immunoprecipitates contained three (glyco)proteins (175, 160 and 95 kDa) (Barbe et al., 1990). However, our subsequent preclearing experiments with specific antibodies showed that the 160 and 95 kDa polypeptides represented contaminating CD11b/CD18 integrin (Mac-1), which was not directly associating with the 175 kDa ED2 antigen (data not shown). Thus, after preclearing the lysate over a column with irrelevant IgG, affinity purification on the ED2 column yielded the major ~175 kDa protein (Figure 2A). Amino acid sequencing of the N-terminus and internal peptides (Table 1) identified the ED2 molecule as the rat ortholog of CD163. The minor band from Figure 2A was confirmed by peptide sequencing to also be rat CD163 (data not shown), probably represents a degradation product of CD163 or a splice- or O-glycosylation- variant. The rat CD163 polypeptide sequence is very similar to its human (Law et al., 1993) and mouse (Schaer et al., 2001) counterparts (for an alignment of the complete predicted rat, mouse and human CD163 amino acid sequences see Figure 4). Rat spleen CD163 is a glycoprotein carrying a significant amount of N-linked glycans, as indicated by the reduction in molecular weight upon PNGase F treatment to ~140 kDa (Figure 2B).

The latter roughly corresponds to the calculated molecular weight of 128,3 kDa of the predicted rat CD163 1184 amino acid polypeptide, although clearly there may be some O-glycosylation as well. Furthermore, rat CD163 migrated at a lower molecular weight (~145 kDa) under non-reducing conditions, suggesting internal disulfide bridges (Figure 2C), which is in line with the presence of SRCR domains that are predicted to have a number of intradomain disulfide bonds. CD163 is a member of the group B SRCR family and has been shown to act as a receptor for the binding and uptake of hemoglobin-haptoglobin (Hb-Hp) complexes (Kristiansen et al., 2001; Madsen et al., 2004; Nielsen et al., 2006). Collectively, these results identify the ED2 antigen as the rat CD163 glycoprotein.

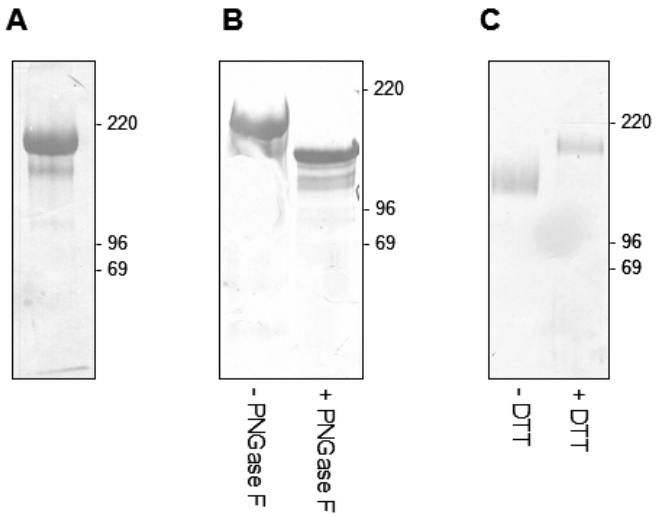

Figure 2: *Purification and identification of the ED2 antigen as rat CD163*

A. The ~175 kDa ED2 antigen was immunoaffinity purified from rat spleen, electrophoresed, blotted and stained with Coomassie Brilliant Blue. **B.** Analysis of N-linked glycosylation of the CD163/ED2 antigen by endoglycosidase F treatment. **C.** SDS-PAGE of CD163/ED2 antigen under reducing (+DTT, 175 kDa) or non-reducing (-DTT 145 kDa) conditions. All samples were run on 10% SDS-PAGE under reducing conditions (+DTT) unless indicated otherwise.

Peptide	**ED2 antigen**	**Rat CD163**	**Position**	**Identity(%)**
1 (N-term.)	VTQAPEGRKKELLLAG	VTQAPEGRKKELRLAG	30-45	94
2	WGTVCDDNFSK	WGTVCDDNFSK	170-180	100
3	QLGCGSALSFSGSAK	QLGCGSALSFSGSAK	189-203	100
4	QLGCPTAITAIGRVNASK	QLGCPTAITAIGRVNASE	300-318	94
5	EDAGVTCSDGADLELR	EDAGVTCSDGADLELR	349-364	100
6	LVGGEIPCSGR	LVGGEIPCSGR	475-485	100
7	GAGQVWRHK	GAGQVWRHK	633-641	100
8	VDTLWQCPSSPWK	VDTLWQCPSSPWK	894-906	100
9	EAAFGPGTGPIWLNEMK	EAAFGPGTGPIWLNEMK	976-992	100

Table 1: *Amino acid sequencing of the N-terminus and internal peptides identified the ED2 molecule as the rat ortholog of CD163*

CD163 mediates erythroblast adhesion

Our previous studies have shown that the binding of foetal liver erythroblasts to CD163-expressing (i.e. ED2-positive) resident bone marrow macrophages can be strongly inhibited using the ED2 mAb (Barbe et al., 1996). Clearly, it was important to confirm that cellular CD163 expression could confer erythroblast binding. Therefore, we initially tested binding of the human erythroblast leukemic cell line K562 to CHO cells ectopically expressing the full-length human CD163 (Figure 3A). As can be seen in Figure 3b, CD163 expression consistently and significantly supported K562 cell binding.

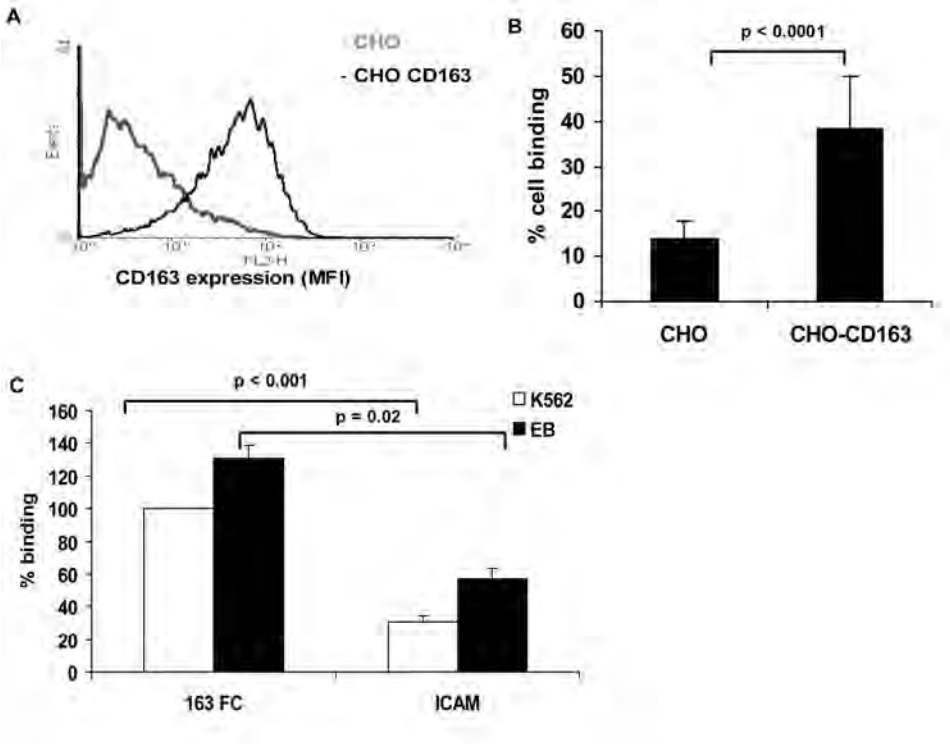

Figure 3: *CD163 functions as an adhesion receptor for erythroblasts*

A. Surface expression of CD163, as identified by FACS staining using mAb EDhu1, on CHO cells stably expressing human CD163 (CHO-CD163) or empty vector (CHO). **B.** K562 erythroblastic leukemic cell binding to CHO cells expressing CD163. Data shown are the means +/- SD from 4 independent experiments. **C.** Binding of K562 cells or freshly isolated rat erythroblasts to fluorescent beads coated with either CD163-Fc or ICAM3-Fc protein. Data shown are the means +/- SD from 3 independent experiments.

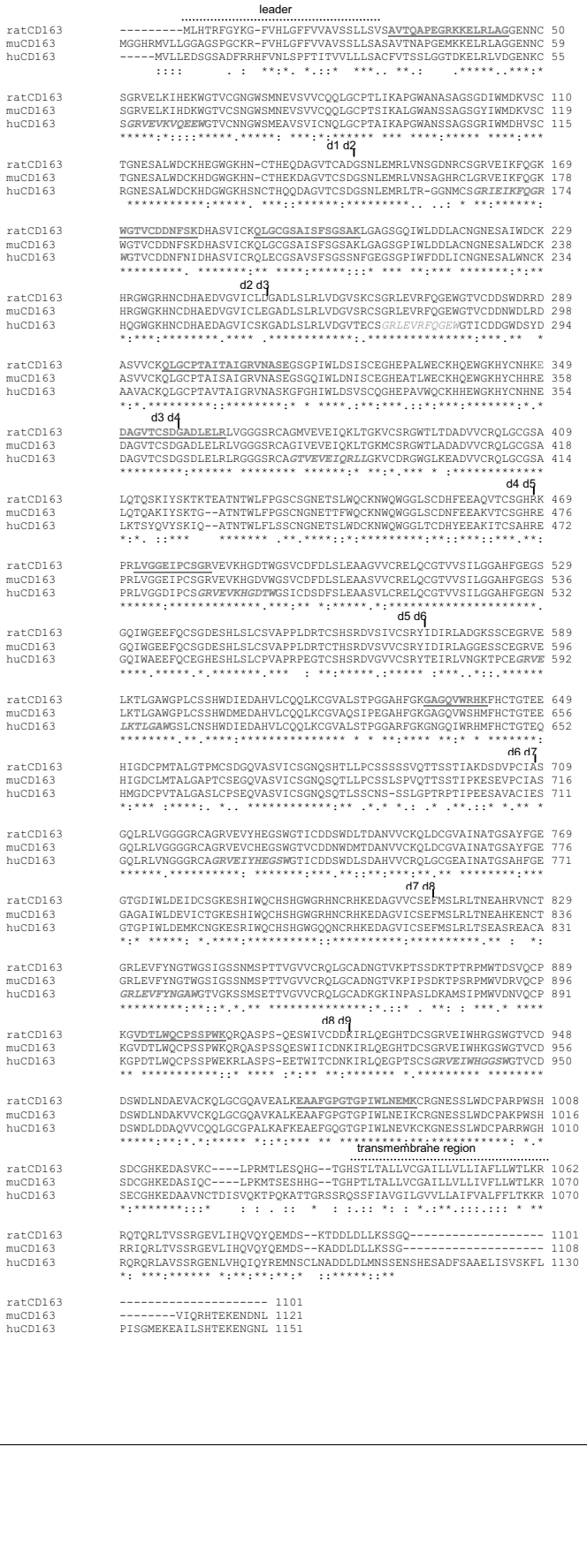
leader
d1 d2
d2 d3
d3 d4
d4 d5
d5 d6
d6 d7
d7 d8
d8 d9
transmembrane region

Figure 4: *The predicted rat CD163 amino acid sequence (XP_232342) and its alignment to mouse (AAK16065)(Schaer et al., 2001) and human (CAA80542)(Law et al., 1993) CD163*

Alignments were performed with the clustalW algorithm version 1.82 and adjusted manually. Amino acid identities for the full-length proteins: rat-mouse 89%, rat-human 74%, and human-mouse 73%. The positions of the peptides identified by amino acid sequencing (Figure 1D) are indicated in grey underlined; the first of these (i.e. VTQAPEGRKKELRLAG) also indicates the N-terminus of the mature polypeptide. The peptides that were synthesized and analysed for erythroblast binding are highlighted in grey italic. Dotted lines indicate respective leader and transmembrane regions; the scavenger domain boundaries are also indicated.

To provide further evidence for a direct interaction between CD163 and erythroblasts we tested binding to a recombinant protein composed of the extracellular region of CD163, including all nine scavenger receptor domains, and the Fc-portion of the human IgG1 molecule (CD163-Fc). When CD163-Fc was coated onto fluorescent beads it resulted in enhanced binding as compared to control beads coated with an irrelevant control protein (ICAM3-Fc) (Figure 3C). The binding of erythroblasts to either CD163 -transfectants or -beads was not significantly affected by using Ca^{2+}-free buffer and/or EDTA (results not shown). Although interactions of SRCR family members are often found to be Ca^{2+}-dependent, divalent cation-independent cell-cell interactions by members of this family have also been reported, for instance for the SRCR class B member CD6 (Patel et al., 1995). These findings, in conjunction with our previously reported ones (Barbe et al., 1996), suggests that CD163 can directly mediate erythroblast adhesion.

An erythroblast adhesion motif in the second scavenger receptor domain of CD163

CD163 is composed of nine extracellular SRCR domains. Previous studies on a closely related glycoprotein gp-340/SAG/DMBT1 have mapped its ligand-binding site to a short amino acid peptide motif in the SRCR domain (Bikker et al., 2002; Bikker et al., 2004). The corresponding peptide in the SRCR domain of MARCO, a SRCR class A family member, has also been shown to mediate ligand binding (Elomaa et al., 1998). Interestingly, molecular modelling (Bikker et al., 2002) using the structure of the Mac-2-binding protein as a template (Hohenester et al., 1999) has localized this 11 amino acid motif to a putative ligand-binding pocket in the SRCR domain. We hypothesized that the corresponding region in one or more of the CD163 SRCR domains may be responsible for erythroblast binding. Therefore, 13-mer peptides were generated encoding these motifs from each of the nine SRCR domains of human CD163 (Figure 5A, see also Figure 2) and tested for binding to K562 cells and freshly isolated rat erythroblasts. Among these CD163 peptides, only the one corresponding to the 11 amino acid motif of the second SRCR domain (CD163p2) of human CD163 displayed specific erythroblast binding (Figure 5B). Binding was dependent on coating concentration, and was also observed when the corresponding peptide of the rat and mouse CD163 domain 2 (p2-rm) was employed (Figure 5C/D). Finally, human erythroblasts (95-98 % pure), generated *in vitro* from human CD34-positive hematopoietic stem cells (Giarratana et al., 2005), also displayed binding to the CD163p2 peptide (Figure 5E). Taken together, these data suggested that the CD163p2

motif was, at least in part, responsible for mediating the CD163 binding to erythroblasts.

Interaction of erythroblasts with the CD163p2 motif promotes erythroid expansion

Upon demonstrating that the CD163p2 motif could contribute to erythroblast adhesion, it was of interest to investigate whether this interaction could also directly affect erythropoiesis. To investigate this possibility, rat erythroblasts were cultured in plates coated with CD163p2 or control peptides. These experiments were performed under conditions, i.e. in the presence of EPO but in absence of other factors, that supports a limited degree of erythroid differentiation and survival, but only allow minimal growth.

By directly counting viable cells, the CD163p2 peptide was shown to specifically stimulate erythroid expansion, as peptides corresponding to the other non-binding CD163 SRCR domain motifs did not (Figure 6A). In contrast, there were no significant effects on erythroid maturation as evaluated by May-Grunwald-Giemsa stainings (Figure 6B). This suggested that binding of erythroblasts to the CD163p2 motif selectively promoted the growth and/or survival of these cells. It should be mentioned that we have not observed the growth promoting effect of CD163p2 peptide under the conditions that support the strong growth of the human CD34-positive hematopoietic stem cell-derived erythroblast cultures. Whether this is directly related to the high level of proliferation already occurring in these cells is not known.

Finally, we investigated whether the adhesion of erythroblasts to CD163p2 is related to their maturation stage. Indeed, *in vitro* maturation of erythroblasts in the presence of EPO (Figure 6C) resulted in a virtually complete loss of CD163p2 peptide binding capacity (Figure 6A). The loss of binding during differentiation coincided particularly well with the disappearance of pro-erythroblasts from the populations, indirectly suggesting that pro-erythroblasts may be the main erythroblast subset responsible for binding to CD163p2.

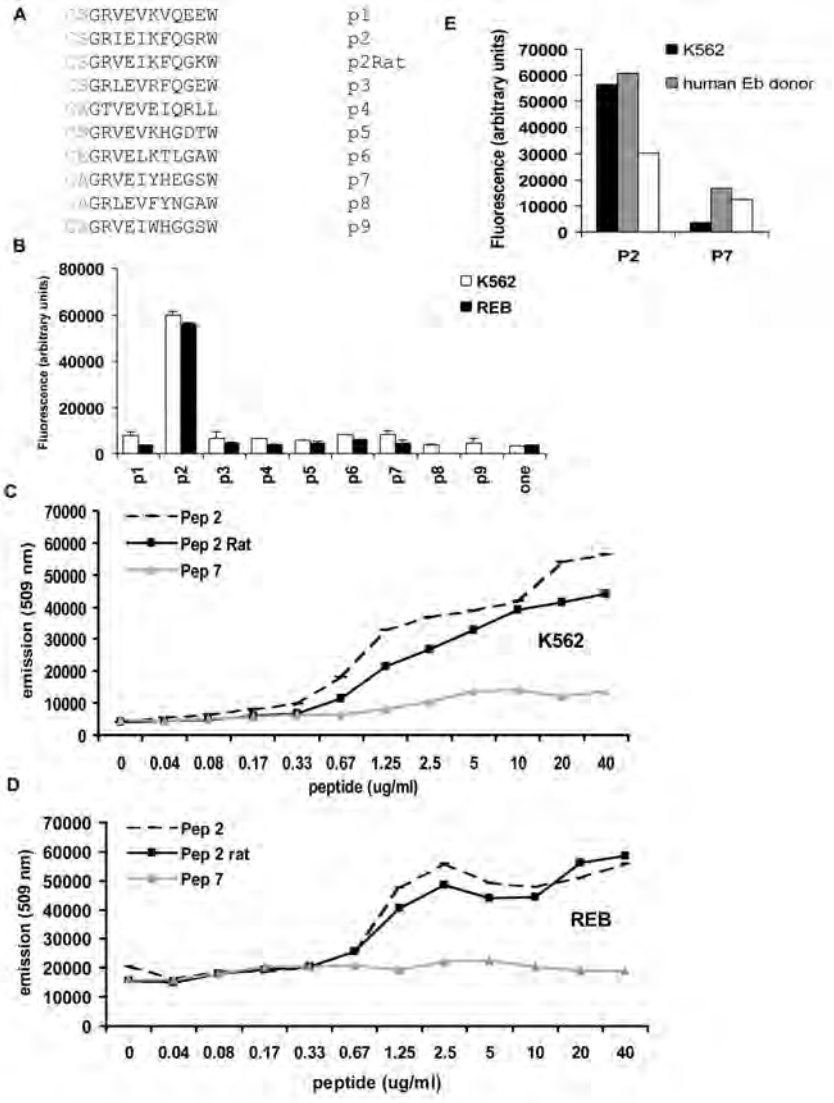

Figure 5: *Identification of an erythroblast-binding motif in the second SRCR domain of CD163*

A. Generated 13-meric peptides corresponding to sequences from each of the nine extracellular SRCR domains of human CD163 (termed p1-p9 respectively) and of the 2nd domain of rat/mouse CD163 (termed p2-rm). **B.** Binding of K562 cells or freshly isolated rat erythroblasts to CD163 peptides. Peptides were used at 40 μg/ml coating concentrations and cell binding was quantified by staining with the DNA-binding dye SYTO-13 followed by measurement of the fluorescence. Data shown are the means +/-SD from at least 5 independent experiments. **C/D.** Concentration dependence of K562 (C) and rat erythroblast (D) adhesion to the human (p2) or rodent (p2-rm) CD163 binding motif. Data shown are from one representative experiment out of 3**. E.** Binding of human erythroblasts, generated *in vitro* from CD34-positive human embryonic stem cells from two separate donors, to CD163 p2 peptide. CD163 p7 peptide is used as a negative control. K562 cell binding is shown for comparison. The two cell populations had comparable numbers (>80%) of erythroblastic cells, with a similar subset distribution.

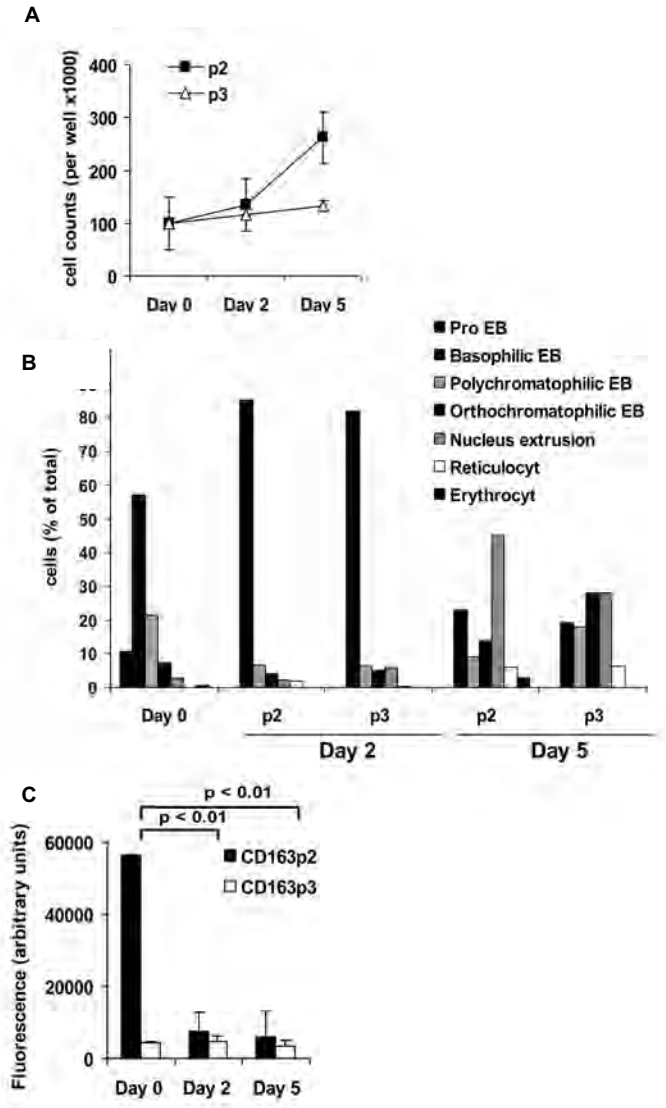

Figure 6: *The CD163p2 motif promotes erythroid expansion*

A. Numbers of viable rat erythroblasts after culture for different times on CD163 peptide p2 and control peptide p3. **B.** Maturation of rat erythroblasts after culture for different times on CD163 peptide p2 and control peptide p3. Results in A. and B. are from one representative experiment out of three. **C.** Binding of rat erythroblasts at different stages after *in vitro* maturation in the presence of 2 IU/ml EPO to CD163 p2 and control p3 peptides. Data shown are the means +/- SD from 3 independent experiments. Statistics: two-tailed T test.

Discussion

In the present report we provide evidence that the surface receptor CD163 can function as an adhesion receptor for erythroblasts. CD163 is highly expressed on macrophages in erythroblastic islands, both in the bone marrow as well as in extramedullary sites of erythropoiesis. We demonstrate that the rat ED2 antigen, which has previously been implicated in macrophage-erythroblasts interactions (Barbe et al., 1996), is the rat CD163 ortholog. Furthermore, we show that CD163 either expressed on cells or as a recombinant protein, can interact directly with erythroblastic cells. We also identify a motif in the second SRCR domain of CD163 that mediates erythroblast binding. Finally, we provide evidence that the interaction of this CD163 motif with erythroblasts promotes the growth and/or survival of these cells. Collectively, this identifies CD163 as a novel adhesion receptor that, together with previously identified molecules and perhaps others (Chasis, 2006), mediates the interaction between macrophages and erythroblasts in erythroblastic islands.

Our findings suggest a potential role for CD163 in erythropoiesis, clearly adding a new dimension to the relationship between CD163 and red blood cells. CD163 has previously been identified as a receptor for Hb-Hp complexes and, as such, is believed to contribute to the clearance of free hemoglobin, a potent oxidant, from the circulation (Kristiansen et al., 2001). This appears to be of particular importance during conditions of intravascular hemolysis, such as autoimmune hemolytic anemia, sickle cell disease or malaria. Consistent with this notion, CD163 is highly expressed on splenic red pulp and liver macrophages (Van den Heuvel et al., 1999; Dijkstra et al., 1985), which are major scavengers, not only of Hb-Hp, but also of senescent red blood cells *in vivo*. Interestingly, our current results (Figure 1) support the idea that these strongly CD163-positive macrophage subpopulations can simultaneously support extramedullary erythropoiesis.

This occurs both during the indicated pathological conditions as described above, which are accompanied by anemia, but also during normal development. Therefore, our findings suggest that CD163 on liver and spleen macrophages could have a dual function, i.e. simultaneously mediating the clearance of Hb and promoting erythropoiesis. Such a possible physical link between Hb clearance and erythropoiesis would provide a very efficient mechanism for recycling iron to developing erythroblasts. Of potential interest in this context, the CD163 Hb-Hp binding site appears to be distinct from the erythroblast-binding site, suggesting that binding of Hb-Hp complexes and erythroid precursors is coordinated by CD163 in erythroblastic islands. In particular, the third SRCR domain of CD163 is most likely involved in Hb-Hp binding (Madsen et al., 2004; Kristiansen et al., 2001), while our current data implicate a motif in the second SRCR domain in erythroblast binding. Consistent with this hypothesis, we have not been able to demonstrate significant inhibition of erythroblast binding to CD163 with antibodies (i.e. EDHu-1) directed against the third SRCR domains (data not shown), which have previously been shown to efficiently block Hb-Hp binding to CD163 (Madsen et al., 2004). As our current peptide analysis was limited to sequences corresponding to the previously identified ligand binding site in the related SRCR class

B family member gp-340/SAG (Bikker et al., 2004; Bikker et al., 2002), we cannot exclude that CD163 domains other than the CD163p2 motif contribute to erythroblast binding.

A recent study (Matthews et al., 2006) has suggested that CD163 is expressed on the surface of a small subset (~2%) of CD34-positive hematopoietic stem cells. Furthermore, evidence was reported that antibodies against the third SRCR domain of CD163 (i.e. EDHu-1, Mac2-158) can promote erythroid expansion from CD34-positive cells in the presence of EPO and IL-3, suggesting that CD163 signaling may somehow promote erythropoiesis. This effect was postulated to mimic previously reported stimulatory effects of Hb on erythropoiesis (discussed in Matthews et al., 2006). Our current findings may suggest an alternative explanation for these results. One possibility to consider, and re-evaluate, is that the small fraction of surface CD163-positive cells within the CD34-positive population may actually be macrophages. If so, then the ligation of CD163, by either Hb-Hp and/or erythroblasts, could induce the generation of erythroid differentiation-promoting factors by macrophages. In line with this idea, other ligands such as EDHu-1 and Hb-Hp have previously been shown to trigger hematopoietic- and inflammatory- cytokine (e.g. GM-CSF, IL-1β, IL-6, IL-10) production in CD163-expressing macrophages (Van den Heuvel et al., 1999; Philippidis et al., 2004). In addition to this, our current data suggest that erythroblasts have a putative ligand for CD163 that specifically recognizes the CD163p2 motif and provides signals that stimulate erythroblasts growth and/or survival. The interactions of CD163 on macrophages with this putative ligand on erythroblasts could therefore regulate erythropoiesis by mediating bi-directional signaling in erythroblastic islands.

Our identification of CD163 as an erythroblast adhesion receptor also constitutes the first evidence for a role of CD163 in cell-cell interactions in general. This corresponds to the proposed functions of several other members of the SRCR class B family, including the lymphocyte surface molecules CD6 (Bowen et al., 1995; Patel et al., 1995; Aruffo et al., 1997) and WC1 (Ahn et al., 2002), and the soluble macrophage molecule Spα (Gebe et al., 2000), which have also been shown to mediate interactions with cell-surface ligands. In particular, the third SRCR domain of CD6 binds to the activated leukocyte cell adhesion receptor (CD166) on endothelial cells (Bowen et al., 1996), and WC1 interacts with an unknown ligand via its SRCR domains 9 and 11 (Ahn et al., 2002). In this context, it will be of interest to evaluate the role of CD163 in interactions between macrophages and other hematopoietic or non-hematopoietic cells as well.

Collectively, these findings show that the macrophage scavenger receptor CD163 can function as an erythroblast adhesion receptor in erythroblastic islands and suggest that this interaction may be instrumental in the regulation of erythropoiesis. Major future challenges will be to identify the natural ligand(s) for CD163 on erythroblasts and to establish the exact role of this interaction in erythropoiesis *in vivo.*

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10.

The macrophage scavenger receptor CD163 functions as an innate sensor for bacteria

In preparation

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Abstract

Background: The surface glycoprotein receptor CD163 belongs to the scavenger receptor cystein-rich superfamily type B (SRCR-B) and is highly expressed by most subsets of mature tissue macrophages *in vivo*. It has previously been identified as an endocytic receptor for hemoglobin-haptoglobin complexes and is believed to contribute to the clearance of toxic free hemoglobin. Here, we demonstrate that CD163 can function as a macrophage receptor for bacteria. CD163 either expressed on cells or as an immobilized recombinant fusion protein, was able to bind to Gram-positive and Gram-negative bacteria. A motif (GRIEIKFQGRW) in the second SRCR domain of CD163 was identified to mediate bacterial recognition. Binding of bacteria to CD163 expressed in monocytic cells induced a potent TNF-α response. Finally, novel antagonistic antibodies against CD163 were able to inhibit cytokine production by freshly isolated human monocytes. These results suggest that CD163, which is broadly expressed on tissue macrophages, acts as a molecular sensor for bacteria and an inducer of innate immunity during bacterial infection.

Introduction

The recognition of pathogens by macrophages plays a critical role in (innate) immunity and is partially mediated by so called pattern-recognition receptors (PRR) (Janeway, Jr., 1992). PRR interact with relatively invariant pathogen-associated molecular patterns (PAMP) on pathogens, such as certain peptidoglycans, lipopolysaccharides, and (modified) nucleic acids. Various classes of PRR have been described on macrophages including members of the C-type lectin families, scavenger receptors, and Toll-like receptors. Many of these mediate the phagocytosis and degradation of pathogens, but some also trigger the production of inflammatory mediators that in turn establish an early-induced response (Janeway, Jr. and Medzhitov, 2002). The expression and contribution of the various PRR depends on macrophage subpopulation and activation states (Gordon et al., 1995). Here, we will focus on one of the PRR, CD163 a scavenger receptor family member that is expressed on most subsets of myeloid cells. Mature tissue macrophages express high levels of CD163, as for instance, Kupffer cells in the liver, red pulp macrophages in the spleen, and cortical macrophages of the thymus (Van den Heuvel et al., 1999).

CD163 is a glycoprotein receptor. It has been demonstrated to function as a receptor for hemoglobin (Hb)- haptoglobin (Hp) complexes (Kristiansen et al., 2001) and is believed to mediate the clearance of free Hb, as such protecting against oxidative tissue damage during hemolysis (Moestrup and Moller, 2004). CD163, which is expressed on resident bone marrow macrophages, also functions as an erythroblast adhesion receptor and promotes the expansion of erythroid precursor cells (Chapter 9). Of interest, cross-linking of CD163 triggers the production of inflammatory mediators, such as nitric oxide, TNF-α, IL-1β, IL-6 and IL-10, suggesting a potential immune modulatory role (Van den Heuvel et al., 1999; Madsen et al., 2004; Philippidis et al., 2004) (Chapter 8).

CD163 is a member of the scavenger receptor cysteine-rich (SRCR) family class B. Related proteins belonging to this family include CD5, CD6, Spα, gp-340 (DMBT-1), and M160 (Sarrias et al., 2004) (reviewed in Chapter 3). The salivary glycoprotein gp-340 was previously identified as a bacterial agglutinin, and an 11 amino acid motif within its SRCR domain was implicated in bacterial recognition (Bikker et al., 2004). More recently, the secreted Spα molecule was also described to bind to bacteria, including both Gram-positive and Gram-negative species (Sarrias et al., 2005). The bacterial interaction site of the Spα molecule was determined to be located within the first scavenger domain.

In the present report we demonstrate that CD163 functions as a macrophage receptor for bacteria. The extracellular domain of CD163, expressed either on cells or as a recombinant protein, directly supported the binding of bacteria, including both Gram-negative and Gram-positive species. Binding could be mimicked using a peptide motif corresponding to the second SRCR domain of CD163, suggesting that this domain harbors a binding site for bacterial binding. Recognition of bacteria by CD163 potently enhanced inflammatory cytokine production in monocytic cell lines and cytokine production by freshly isolated human monocytes was strongly suppressed using novel

antagonistic monoclonal antibodies against CD163 that prevented bacterial binding to CD163.

Materials and Methods

Reagents

Peptides were generated by solid phase peptide synthesis on a Milligen 9050, Pepsynthesizer (Millipore, Milford, MA, USA) as described by Bikker and coworkers (Bikker et al., 2002). The purity of the peptides was indicated at least 85%. Lipopolysaccharide (LPS), derived from Escherichia coli strain 055:B5 was obtained from Difco (Detroit, MI).

For human anti-CD163 antibodies we used EDHu-1 which was developed at our own laboratory (Van den Heuvel et al., 1999) and commercially available via Serotec (Oxfordshire, UK), RM3/1 (Biomedicals AG, Augst, Switzerland), and Mac 2-158 (Maine Biotechnology Services, Portland, ME).

Anti-CD14 PE (clone M5E2) was obtained from BD Bioscience Pharmingen (San Diego, CA). Anti-CD68 (clone KP1) was obtained from DAKO (Copenhagen, Denmark) and anti-CD15 PE and anti-CD11b PE from BD Bioscience Pharmingen (San Diego, CA). As a control IgG_1 human anti HCG was used.

Bacteria

S. mutans (Ingbritt), *E. coli* (K4), *S. aureus* (clinical isolate), and *E.coli Dsred* (van der Sar et al., 2003) were cultured on blood agar plates under anaerobic conditions with 5% CO_2 for 24 h at 37 °C. Bacteria were harvested and washed twice in Tris-buffered saline (TBS-Ca^{2+}: 10 mM Tris, pH 7.5, containing 150 mM sodium chloride and 2 mM calcium chloride). Bacteria were adjusted to their final concentration by measuring the optical density (OD) at 600 nm. Unless indicated otherwise viable bacteria were used. In some cases UV-killed bacteria were used. UV killing of bacteria was performed by exposing bacteria (diluted to 1 10^9 bacteria/ml) to UV light for 30 min. Bacteria were stored at −20 °C until further use.

Bacteria were labelled with fluorescein 5-isothiocyanate (FITC, isomer I, Molecular Probes, Eugene, OR) by a modification of the procedure of (Hazenbos et al., 1994). Bacteria from overnight cultures were suspended into TBS-Ca^{2+}, and adjusted to an A_{600} of 1 (corresponding to about 1×10^9 bacteria/ml). One ml of bacteria was transferred to a microcentrifuge tube, pelleted, and resuspended in 1 ml of FITC (0.5 mg/ml) in TBS-Ca^{2+}. Bacteria were incubated for 1 hr at 37 °C, washed at least three times in TBS-Ca^{2+} at 4000 rpm for 7 min at 4°C, and then suspended in 660 μl TBS-Ca^{2+}.

Generation of CD163 expressing THP-1 cells

The full-length DNA sequence encoding full length human CD163 was excised from a pCDNA3.1-based plasmid (Kristiansen et al., 2001) using KPN I and Not I and cloned into pBacPAK-His1 and subsequently into the modified retroviral vector LZRS-IRES-GFP (Kinsella and Nolan, 1996) by using Xho I and Not I. The resulting construct, LZRS-

CD163-IRES-GFP, was transfected using calcium phosphate into amphotropic Phoenix retrovirus producer cells (Kinsella and Nolan, 1996) for the generation of helper free amphotropic retroviruses. Virus-containing supernatant was used to transduce THP-1 cells in a plastic culture dish pre-treated with 30 μg/ml retronectin (Takara Biomedicals, Shiga, Japan). After 48hr at 32°C, cells were transferred to fresh medium. Transduction was repeated 3 times until approximately 5% of the THP-1 cells expressed CD163-IRES-GFP. GFP expression was determined by flow cytometry on a FACS Calibur (Becton & Dickinson, San Jose, CA, USA). Cells were sorted on GFP expression by flow cytometry cell sorting (MoFlo, DAKO Cytomation cell sorter) and enriched until THP-1 CD163-GFP cells were 95%-100% GFP positive. As a control an empty vector (EV) LZRS-IRES-GFP construct was introduced into THP-1 cells. Cells were kept in culture in RPMI medium (PAA, Pashing, Austria) containing 10% fetal calf serum (Bodinco, Alkmaar, The Netherlands) and antibiotics (penicilline/streptomycine (Cambrex, Verviers, Belgium)) and CD163 expression was checked regularly.

Monocyte isolation and cell culture

Peripheral blood mononuclear cell (PBMC) from healthy donors, obtained with informed consent, were isolated by Ficoll-IsoPaque density gradient centrifugation. Subsequently, cells (2.5 10^8 cells/ml) were resuspended in PBS+EDTA+1%FCS and incubated with 50 μl/ml of anti-CD14 MACS magnetic beads in the MACS column purification system (Miltenyi Biotec, Bergisch Gladbach, Germany) for 30 min at 4°C. The MACS column was washed with 3 ml of MACS buffer before addition of the respective sample. PBMC coupled to MACS beads were eliminated from the samples by retention in the column in a magnetic field. Monocytes were stored at –80 °C until further used. After thawing, cells were resuspended in RPMI medium (PAA) supplemented with 10% fetal calf serum and antibiotics, and cultured for 2 days with or without 10^{-6} M dexamethasone (DEX; Sigma, St Louis, USA).

Flow cytometry

CD163 expression of the THP-CD163-GFP cells and the stabely transfected CHO-CD163 cells (Kristiansen et al., 2001) was determined on 100.000 cells using monoclonal anti-human CD163 (EDHu-1) and anti-human CD68 (KP1). Cells were stained with the monoclonal antibodies (10 μg/ml) for 60 min at 4°C in PBS-0.1% bovine serum albumin (BSA; Boehringer-Mannheim, Mannheim, Germany), and washed. Subsequently, cells were stained with goat-anti-mouse ALEXA Fluor 633 (Molecular Probes, Invitrogen, The Netherlands) for 45 min. For staining with CD14PE, CD15PE, and CD11b PE cells were incubated with the direct conjugates antibodies for 45 min. After washing, cells were resuspended in PBS-0.1% BSA and fluorescence intensity was determined using a FACS-calibur flow cytometer (Becton and Dickinson, San Jose, CA, USA).

Antibody generation

BALB/c mice were immunized 3-4 times with 5-10 10^6 stably CD163 transfected CHO cells using an immunization protocol as described previously (Dijkstra et al., 1985). Spleen cells were then fused with the mouse myeloma cell line Sp2/0 and the resulting hybridomas were plated at a density of ~1 cell/well under hypoxanthine aminopterin thymidine selection. Hybridoma supernatants were collected and screened for specific reactivity to CD163 CHO cells and subsequently to human spleen macrophages with immunohistochemistry (see below). Selected hybridomas were subcloned by limiting dilution. Cytospin preparations of CD163 CHO cells and CHO WT cells, and 8–9 µm snap frozen human spleen tissue sections were prepared and air dried on gelatin-coated slides. Slides were fixed for 10 min in acetone and washed in PBS. Preparations were incubated with 100 µl hybridoma supernatant from monoclonals for 45–60 min at RT. Subsequently, preparations were washed twice and incubated with horseradish peroxidase-conjugated rabbit anti-mouse Ig (DAKO A/S, Glostrup, Denmark) for 45–60 min. Staining was visualized using 3,3'-Diaminobenzidine (Sigma)/H_2O_2.

Cell binding assays

CD163 CHO cells and WT CHO cells ($2.5x10^5$ cells) were incubated with 100 µl of FITC-labelled bacteria (corresponding to $2.5x10^6$ bacteria) in TBS-Ca^2 at 4°C for 30 min. Samples were washed 3 times (1400 rpm for 3 min) and analyzed by flow cytometry.
For the non-adherent THP-1 cells the following assay for analyzing the binding of bacteria was performed. THP-EV-GFP and THP-163-GFP cells ($1x10^5$ cells in 100µl TBS-Ca^{2+}) were incubated with DsRed-transfected *E.coli* (at a ratio of 100 bacteria/cell) and incubated for 1 hr at 4°C. Samples were washed 3 times (1400 rpm for 3 minutes) and analyzed by flow cytometry. Where indicated antibodies against CD163 or isotype matched controls were pre-incubated with the cells and washed twice before bacteria were added.

Solid phase adhesion assay

Solid phase binding of bacteria to synthetic peptides was performed as described previously (Bikker et al., 2002). Briefly, 40 µg/ml of synthetic peptide was diluted serially in coating buffer (100 mM sodium carbonate, pH 9.6) in microtiterplate (Fluotrac 600; Greiner, Recklinghausen, Germany). After overnight incubation at 4°C plates were washed and 100 µl bacteria-suspension (5 10^8 bacteria/ml in TBS-Ca^{2+} containing 0.1% Tween 20) was added and incubated for 2 hr at 37° C. After washing with TBS-Ca^{2+} containing 0.1% Tween 20, adhesion of bacteria was quantified by labelling with the cell-permeable DNA-binding probe SYTO-13, (1:1000, Molecular Probes, Leiden, The Netherlands) and measured in a fluorescence microplate reader (Fluostar Galaxy, BMG Laboratories, Offenburg, Germany) at 488 nm excitation and 509 nm emission.

Bacterial aggregation assay

The bacterial aggregation assay was performed as follows. A suspension of bacteria (150 μl; $5x10^8$ bacteria/ml TBS-Ca^{2+}) was mixed with 150 μl peptide solution (at final concentrations of 0-200 μg/ml in TBS-Ca^{2+}) for 2 hr at 37° C. After agglutination 10 μl of sediment was transferred to a microscopic slide. After heat fixation, bacteria were stained with a 20% crystal violet solution (Merck) and examined by light microscopy.

Fluorescent bead adhesion

CHO cells were stably transfected with a plasmid to express the extracellular domain of CD163 (amino acids 1-993) as a fusion protein with human IgG1 Fc (generously provided by dr. R.J.J. van Neerven). Supernatants from these cultures were collected and CD163-Fc derived from stably transfected CD163 CHO cells was purified before coating onto carboxylate-modified TransFluoSpheres (488/645 nm, 1.0 μm, Molecular Probes, Eugene, OR) as described previously (Geijtenbeek et al., 1999). Briefly, streptavidin was covalently coupled onto TransFluoSpheres as described by manufacturer. To enable coupling of CD163-Fc, streptavidin-coated beads were allowed to bind to biotinylated goat-anti human anti-Fc Fab_2 fragments (6 μg/mL, Jackson ImmunoResearch Lab. Inc.) in 0.5 mL TBS containing 2 mM Ca^{2+} for 2 hr at 37°C. The beads were washed once with TBS-Ca^{2+} and incubated with human CD163-Fc (1 μg) for 2 days at 4°C. The ligand-coated beads were washed, resuspended in 100μL TBS-Ca^{2+}, and stored at 4°C. As a control human ICAM-3-Fc (Van Kooyk et al., 1996) coated beads were used.

For bead adhesion to bacteria, bacteria were resuspended in TBS-Ca^{2+} in a concentration of $1x10^9$ bacteria/ml. Screening for blocking activity of monoclonal antibodies, of bead adhesion to bacteria, was performed by pre-incubation of beads with 1 ml hybridoma supernatant for 30 minutes at 37°C, then beads were washed twice with TBS-Ca^{2+} by centrifuging at 14000 rpm for 3 min. The ligand-coated beads (1 bead/20 bacteria) were added to 50μl bacteria-suspension and incubated for 60 min at 37 °C.

CD163 mediated adhesion of bacteria was measured by flow cytometry using the FACS-calibur (Becton Dickinson & Co, Oxnard, CA). Unbound beads were excluded from the forward sideward scatter 20,000 events were counted.

Cytokine measurements

THP-CD163-GFP cells, THP-EV-GFP cells, and monocytes cultured in the presence or absence of Dexamethasone were cultured, in triplicate, in a concentration of $1x10^5$ cells/ml into 96-well tissue culture plates. Bacteria were added to the cells at a ratio of 100:1. The supernatants were harvested after 4 hr of culture and stored at -20°C until further use. Where indicated antibodies against CD163 or isotype matched controls were added to the wells. TNF-α concentrations were determined by enzyme-linked immunosorbent assays (ELISA) (BioSource International Inc.). The lower detection limit of the assay ranged from 1 to 10 pg/ml.

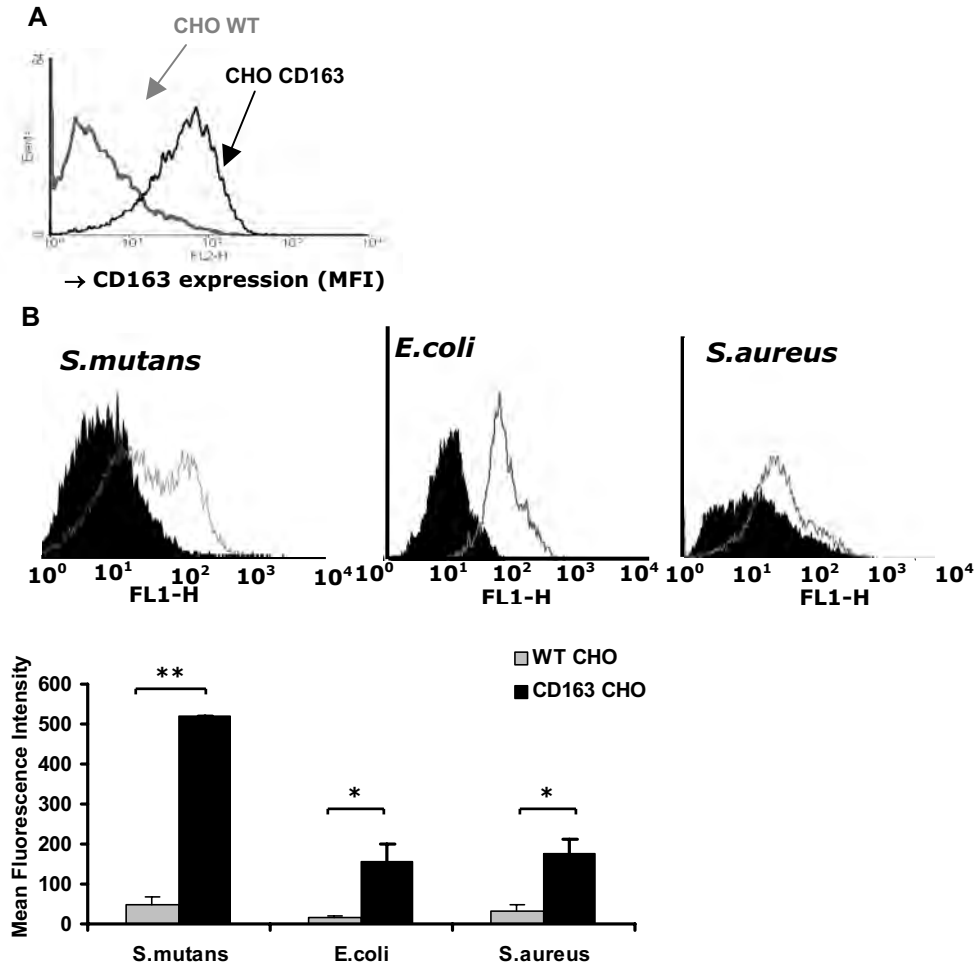

Figure 1

A. CD163 surface expression on transfected CHO cells stained with EDHu-1. Stably transfected CD163 CHO cells (CHO CD163) show a higher mean fluorescence intensity (MFI) than control transfected CHO cells (CHO WT), indicative of high CD163 expression on CHO CD163. **B.** Adhesion of FITC-labeled bacteria to CHO CD163 and CHO WT. CHO CD163 show a higher MFI compared to CHO WT after adhesion of *S.mutans, E.coli, and S.aureus*. In the upper panel a representative flow cytometry plot of the different bacterial strains is given, similar settings were used for all bacterial strains. The filled histogram plot represents CHO WT, whereas the grey line (overlay) represents CHO CD163. The bottom panel shows the median (= standard deviation (SD)) of the MFI of 3 independent experiments. This figure shows that FITC-labelled bacteria bind with higher affinity to CD163 transfected cells (*S.mutans*, $p < 0.0001$; *E.coli*, $p < 0.05$; *S.aureus*, $p < 0.05$, two-tailed T test).

Results

CD163 acts as a bacterial binding receptor

CD163 belongs to the SRCR class B family recently two members of this family, gp-340 and Spα, have been shown to mediate recognition of bacteria (Bikker et al., 2002; Sarrias et al., 2005). We investigated whether CD163 would also be able to mediate binding of Gram-positive and Gram-negative bacteria. First, we employed a flow cytometric assay to test the binding to CD163-expressing CHO cells of various FITC-labelled species of bacteria, including Gram-positive *Streptococcus mutans and Staphylococcus aureus,* and Gram-negative *E. coli* (Figure 1A). CHO cells transfected with empty vector were used as a control. For all of these strains binding to CD163-expressing cells clearly exceeded that seen with wild-type (WT) CHO cells (figure 1B). It should be noted that, although the ratios of binding between CD163 expressing and WT CHO cells may give some idea about the extend of binding of CD163 to a particular bacterial strain; a direct comparison is hampered by intrinsic difference in FITC labelling capacity between the various bacteria. These experiments indicated that cell surface CD163 might support the recognition of bacteria.

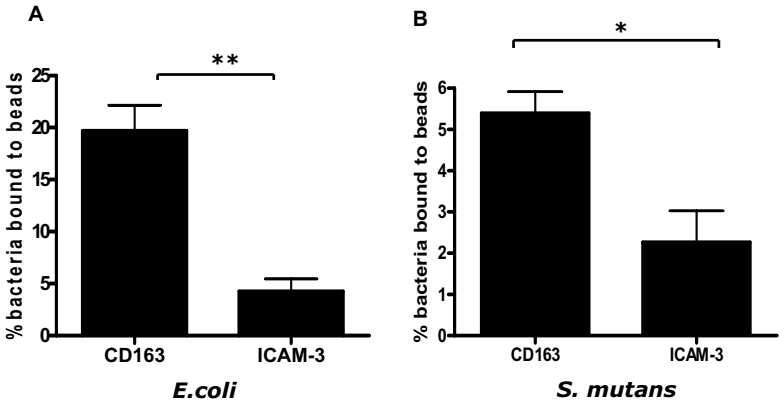

Figure 2: *Bead adhesion assay of CD163-streptavidin coated beads and irrelevant protein coated beads (ICAM-3)*

A. with *E.coli*. Bars represent mean value (+SD) of 3 independent experiments. CD163-beads bind *E.coli* with higher affinity than the control-beads ($p = 0.001$, two-tailed students T test). **B.** with *S.mutans.* Bars represent mean value (+SD) of 2 independent experiments. CD163-beads bind *S.mutans* with higher affinity than the control-beads ($p = 0.01$, two-tailed students T test). The lower values for *S.mutans* binding are caused by a difference in flow cytometer settings.

To provide further evidence for a direct interaction between CD163 and bacteria, we tested binding to a recombinant CD163 protein composed of the 9 extracellular SRCR domains of CD163 fused to the Fc-portion of human IgG1 (CD163-Fc (Chapter 9). CD163-Fc was coated onto fluorescent beads, to create a high-density ligand, and these were used to test bacterial binding by flow cytometry. Again, as compared to the control i.e. ICAM-3-coated beads, the CD163 coated beads mediated superior binding to both *S.mutans* and *E.coli* (Figure 2A and 2B). This indicated that CD163 can directly mediate bacterial recognition. Several monoclonal anti-CD163 antibodies were tested for blocking activity of bacterial binding to CD163 (EDHu-1, RM3/1, and Mac 2-158) however none of these showed blocking.

A bacterial binding motif in the second SRCR domain

CD163 is composed of nine extracellular scavenger receptor domains, a previous study on a closely related glycoprotein gp340/SAG has mapped a bacterial binding site to an 11 amino acid peptide motif within the scavenger domain(s) (Bikker et al., 2004). We synthesized the respective 11 amino acid peptides (sequences indicated in chapter 9) corresponding to each of the nine SRCR domains of human CD163 and tested them for binding to *S. mutans*. Among these CD163 peptides the peptide corresponding to domain 2 (CD163p2) displayed high *S.mutans* binding (Figure 3A). Of interest, we have recently shown that the CD163p2 motif can also mediate erythroblast binding (Chapter 9). In contrast, Hp-Hb binding is most likely mediated by the third domain of CD163 (Kristiansen et al., 2001).

The corresponding peptide from gp-340 previously been described to mediate bacterial aggregation (Bikker et al., 2002). We tested whether CD163p2 could also induce bacterial aggregation. Figure 3B shows aggregation of *S.mutans* in the presence of CD163p2. At increased calcium levels, lower peptide concentrations were required for aggregation, whereas EDTA showed similar results as buffer with 1mM Ca2+. No aggregation was observed with any of the 8 peptides representative of the other CD163 domains (CD163p1, CD163p3-CD163p9). These findings suggest that the CD163p2 motif mediates bacterial binding.

Recognition of bacteria by CD163 in monocytes triggers inflammatory cytokine production

Previous studies have demonstrated that the ligation of CD163 on monocytes, using either monoclonal antibodies or Hp-Hb, results in the secretion of (pro)inflammatory cytokines (Van den Heuvel et al., 1999; Philippidis et al., 2004) In order to investigate whether recognition of bacteria by CD163 also induces a cytokine response in monocytic cells we designed a cell-based system. We introduced CD163 by retroviral transduction into human monocytic THP-1 cells, which express little or no endogenous CD163 (Figure 4A). As a control we transduced THP-1 cells with an empty GFP-containing vector. Apart from a clear shift in CD163 surface expression the introduction of CD163 did not have a major impact on the phenotype of these cells (Figure 4A). In line with the above the CD163-expressing THP-1 cells (THP-CD163-GFP) displayed a mildly enhanced binding of Ds-Red-expressing *E. coli* (Figure 4B).

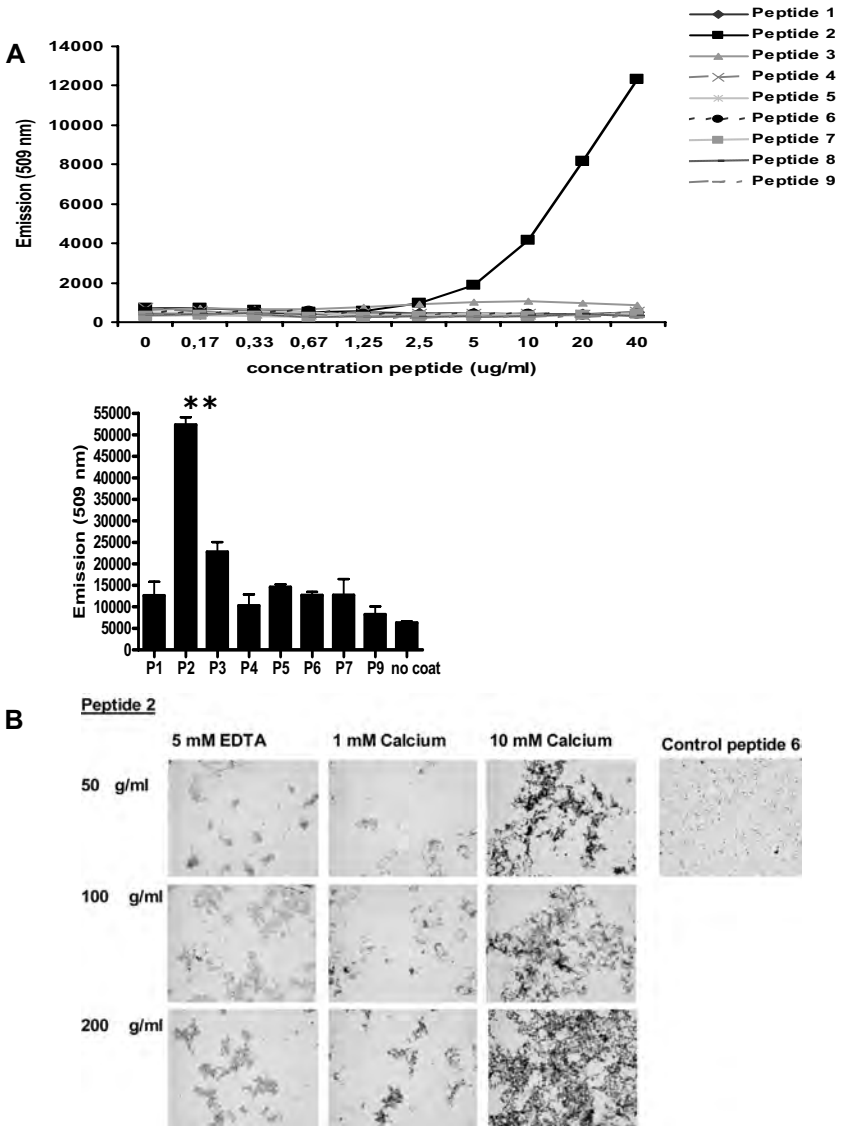

Figure 3

A. Binding of *S.mutans* to 11-mer peptides corresponding to the extracellular scavenger domains 1-9 (for peptide sequences see Table 1). Bacterial binding was quantified by staining with the DNA-binding dye SYTO-13 followed by measurement of the fluorescence. The **upper panel** shows a representative experiment of a serial dilution of all peptides adhered to *S.mutans*. It is clear that peptide 2 binds with high affinity to *S.mutans* to a concentration of 2.5 μg/ml, whereas other peptides show a background signal. In the **bottom panel** shows combined data from 3 independent experiments for *S.mutans* with all peptides. Peptides were used at 40 μg/ml coating concentrations. Data shown are the means + SD. Statistics: two-tailed T test ($p < 0.0001$). **B.** Peptide 2 induces aggregation of *S.mutans*. Peptide 2 was added in various concentrations (50, 100, 200 μg/ml) to *S.mutans* in the presence of 5mM EDTA, 1mM calcium, and 10 mM calcium. In the presence of 10 mM aggregation was stronger and started at lower concentrations than with 1mM calcium or EDTA. All other peptides tested did not agglutinate *S.mutans* (peptide 7 is given as a representative example). These experiments were performed in triplicate.

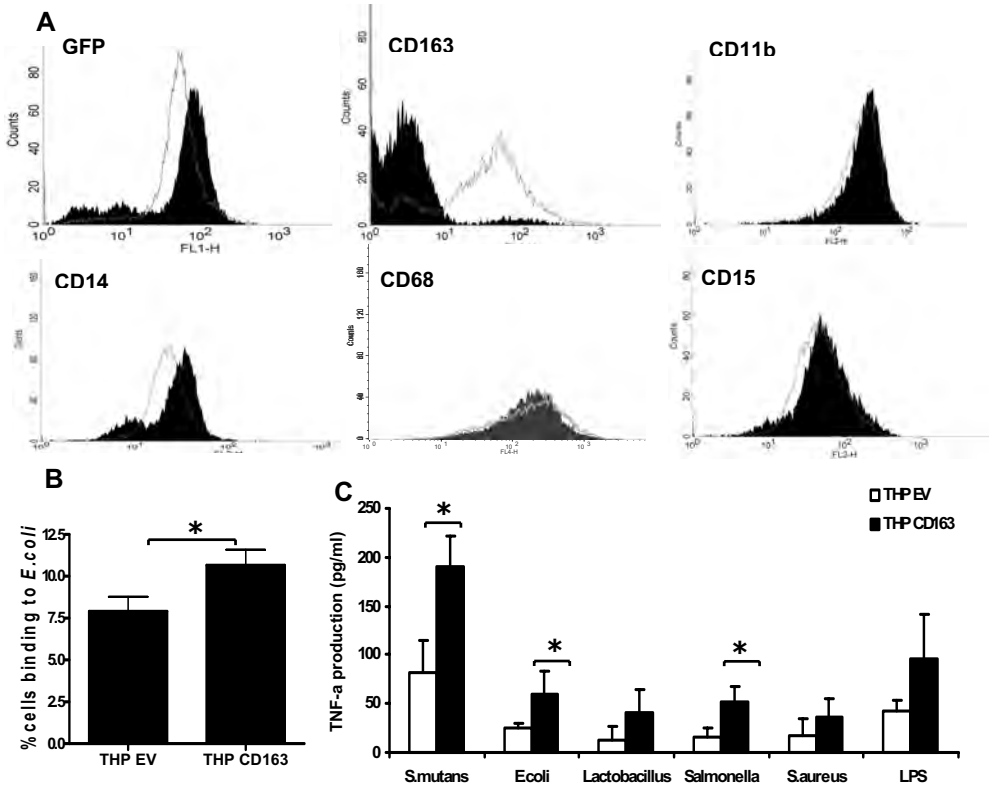

Figure 4

A. Validation of the stably transduced THP cell line, cells were transduced with either EV-IRES-GFP or CD163-IRES-GFP. Cells were stained with CD11b, CD15, CD14, and CD68 and MFI was established by flow cytometry to confirm unchanged phenotype of THP cells after transduction. Filled histograms represent THP-GFP cells whereas the grey line represents the THP-CD163-GFP cells. Furthermore, both EV and CD163 cells showed relative high GFP expression and THP-CD163-IRES-GFP cells showed positive EDHu1 staining, to confirm CD163 expression. **B.** THP-CD163-GFP cells show a higher adhesion of *E.coli* than THP-EV-GFP cells. DsRed transfected *E.coli* were added to THP cells and incubated for 1 hr. After washing, fluorescence was measured in the flow cytometer. An increase in MFI indicates an increase in the amount of bacteria adhered to the cells. THP-CD163-GFP cells show a higher binding of *E.coli* than THP-EV-GFP cells ($p < 0.05$, two tailed t test). Bars represent mean value (+SD) of 3 independent experiments. **C.** Production of TNF-α by transduced THP-cells after incubation with *S.mutans, E.coli, Lactobacillus, Salmonella, S.aureus,* and LPS (100ng/ml). Bacteria were added in a concentration of 100 bacteria per cell and incubated for 4 hours. THP-CD163 cells show a higher TNF-α production after incubation with *S.mutans* (p value = 0.005), *E.coli* (p value = 0.03), and *Salmonella* (p = 0.007). Bars represent mean value (+SD) of 2 independent experiments (two tailed t test).

The relatively high binding by control THP-1 cells (THP-EV-GFP) is presumably due to the presence and activity of other pattern recognition receptors expressed by these cells. To determine whether binding of bacteria by CD163 resulted in cytokine production the cells were incubated with various strains of viable bacteria (i.e. *S.mutans, E.coli, Lactobacillus, Salmonella, S.aureus*) for 4 hr and the production of TNF-α was measured. A significantly enhanced production of TNFα was observed in the CD163-expressing THP-1 cells in response to *S.mutans, E.coli, and Salmonella.* The other two species of bacteria showed a similar trend, as did LPS from *E.coli* (100ng/ml). The latter would implicate LPS as one of the potential bacterial ligands for CD163, which would be in line with the observed binding of Spα to LPS (Sarrias et al., 2005). Antagonistic antibodies against CD163 inhibit inflammatory cytokine production

Clearly, it was of interest to identify antagonistic antibodies that would prevent the CD163-mediated bacterial adhesion and cytokine production. However, none of several previously described monoclonal antibodies showed significant blocking of bacterial binding or cytokine production (not shown). Therefore we generated a novel set of monoclonal antibodies against human CD163. Hybridomas producing antibodies against CD163 were first selected by staining of CD163-CHO and WT-CHO cells. The CD163-reactive clones were tested for inhibition of bacterial binding or TNF-α production. Clones 15.3B and 15.3C, were shown to significantly inhibit *S.mutans* binding to CD163-coated beads (Figure 5A), whereas clone 12.4 did not block bacterial binding. The binding of *E.coli* to CD163 beads, which appeared somewhat more variable, was not significantly reduced. However, 15.3B and 15.3C also reduced the relatively low level of CD163-dependent binding to THP-CD163 cells (Figure 5B). The two apparently antagonistic mAb were subsequently tested for their effect of bacteria-induced cytokine production in the CD163-expressing THP-1 cells. Of interest, both mAb 15.3B and 15.3C essentially prevented CD163-dependent *E.coli*-elicited TNF-α production (Figure 5C).

Finally, we investigated the effect of the antagonistic mAb on cytokine production triggered by intact bacteria using freshly isolated human monocytes that had been cultured for 2 days in the presence or absence of the glucocorticoid dexamethasone (DEX). A significant inhibition of TNFα production was seen. This was not only observed after prior culture in DEX (Figure 6B), which had as expected resulted in a strongly enhanced surface CD163 expression (data not shown) as frequently reported previously (Hogger et al., 1998; Van den Heuvel et al., 1999), but also occurred in cells cultured in the absence of DEX (Figure 6A) that express relatively low levels of CD163. Although we have at present no information with respect to absolute numbers of CD163 molecules on monocytes, the significant contribution of CD163 to cytokine production in spite of these low expression levels could be indicative of quite a potent signaling capacity upon recognition of bacteria by CD163.

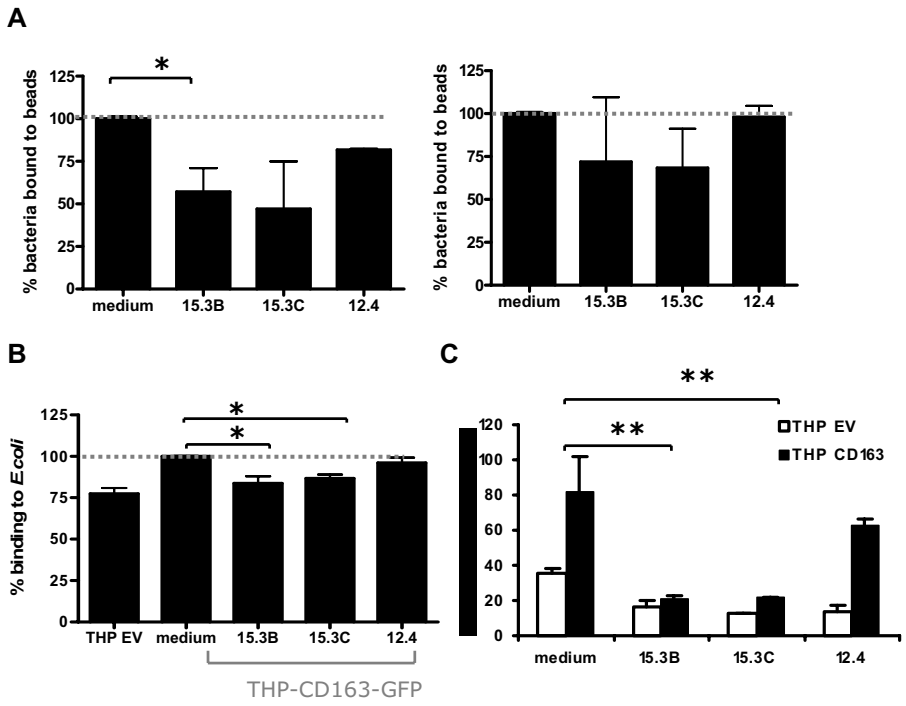

Figure 5

A. Bead adhesion assay of CD163-streptavidin coated beads incubated with *S.mutans* (left) or *E.coli* (right) and blocked with anti-CD163 hybridoma-culture supernatants. Beads were pre-incubated with culture supernatant or medium control for 30 min washed and added to the bacteria. A representative experiment is shown. Results from bacteria binding to CD163-beads pre-incubated with medium were set at 100%. 15.3B shows a significant decrease in binding of CD163 to *S.mutans* (two tailed t test, p value = 0.05) in *E.coli* this was not significance although a clear decrease in binding after pre-incubation with 15.3B and 15.3C can be seen. **B.** Blocking of *E.coli DsRed* to THP-CD163-GFP cells. Cells were pre-incubated with hybridoma-supernatants, washed and bacteria were added (cell: bacteria ratio; 1:100). Bars represent mean value (+SD) of 3 independent experiments. Results from bacteria binding to THP-CD163GFP cells pre-incubated with medium were set at 100%. 15-3B and 15-3C show a significant decrease in binding of CD163 to S.mutans (two tailed t test, p value = 0.03 respectively p = 0.02). **C.** TNF-α production after E.coli adhesion to THP-EV or THP-CD163 cells blocked with supernatants from anti-CD163 hybridoma-culture. A clear reduction in TNF-α production by THP-CD163 cells is seen after incubation with anti-CD163 hybridoma supernatant 15.3B and 15.3C (two tailed t test, p value = 0.001 respectively p = 0.001). However no reduction in TNF-α production by THP-CD163 is seen after pre-incubation with hybridoma 12-4A, moreover this antibody also blocks CD163 specific binding to beads or cells.

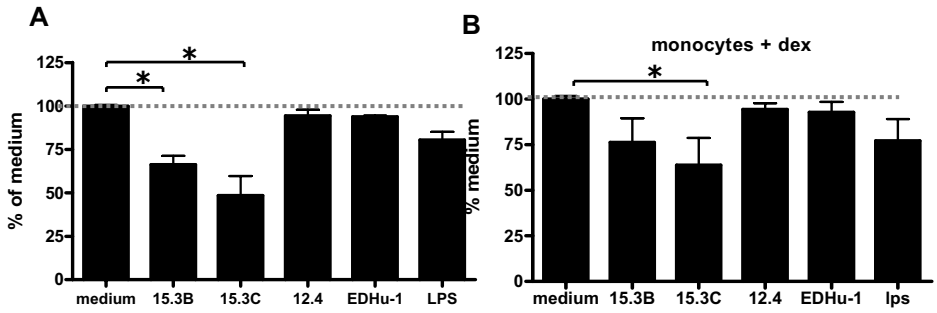

Figure 6: *TNF-α production in human monocytes after S.mutans adhesion blocked with anti-CD163 antibodies*

Data are normalized to vehicle control (medium) to exclude donor variability.
A. A clear reduction in TNF-α production in response to *S.mutans* is seen after incubation with anti-CD163 hybridoma supernatant 15.3B and 15.3C (two tailed t test, p value = 0.01 respectively p = 0.003). Similar results were observed with *E.coli* (experiment has only been performed once). **B.** TNF-α production after *S.mutans* adhesion to DEX stimulated monocytes blocked with supernatants from anti-CD163 hybridoma-culture. A clear reduction in TNF-α production is seen after incubation with anti-CD163 hybridoma supernatant 15.3B and 15.3C (two tailed t test, p value = 0.01). Similar results were observed with *E.coli*.

Discussion

In the present report we demonstrate for the first time that the macrophage scavenger receptor CD163 can mediate recognition of bacteria, including Gram-positive and Gram-negative species. This is supported by the observation that CD163, expressed either on cells or as an immobilized recombinant protein, can specifically mediate bacterial binding. Furthermore, we identify a potential 11 amino acid binding motif in the second SRCR domain of CD163. Finally, we provide evidence that bacterial recognition by CD163 results in a potent cytokine production in monocytic cells and demonstrate that monocyte cytokine production induced by intact bacteria can be inhibited by a set of newly generated antagonistic monoclonal antibodies against CD163. Collectively, these findings support the idea that CD163 on macrophages functions as a typical pattern recognition receptor for bacteria.

CD163 is a member of the SRCR group B family, characterized by the presence of nine SRCR domains. Several other members of this family, including Spα and Gp-340 (Elomaa et al., 1998; Bikker et al., 2002; Sarrias et al., 2005), as well as the somewhat more distantly related class A SRCR family member MARCO (Elomaa et al., 1998) have also been shown to mediate bacterial recognition through their SRCR domains. The recognition of bacteria by the other class A scavenger receptors, SR-AI/SR-AII, appears mediated via the collagenous region instead. One interesting possibility is that bacterial recognition is a common and perhaps ancient feature of SRCR superfamily members. It will be interesting to investigate whether other members of this family, including for instance the lymphocyte CD5 and CD6 surface molecules, also have this property. In this context, another relevant aspect of CD163,

gp-340, Spα, and perhaps also MARCO is that they appear to share a structurally conserved site within the scavenger domain that can mediate bacterial binding. This is represented by an 11 amino acid bacterial recognition consensus motif: GxVEVLxxxxW (Bikker et al., 2004).In MARCO the RxR sequence, which is part of the RGRAEVxxxxxW sequence, appears critical for bacterial recognition (Brannstrom et al., 2002). Of interest, in Mac-2 binding protein the relevant motif appears exposed in a putative binding loop within the scavenger domain, which thus far is the only SRCR superfamily member for which a structure has been reported (Hohenester et al., 1999). In the case of CD163, which has nine SRCR domains, we have found that bacterial binding is selectively mediated by the peptide motif from the 2nd SRCR domain, and perhaps also, but certainly to a lesser extend, by that from the 3rd domain. Of interest, the same motif from domain 2 also appears to mediate the previously reported adhesion of erythroblasts (Chapter 9). Available, mostly indirect, evidence suggests that the binding of Hp-Hb complexes may rather involve the 3rd SRCR domain (Madsen et al., 2004). The notion that CD163 employs at least two structurally different binding sites to interact with its various ligands is supported by the observation that some monoclonal antibodies, such as EDhu1 directed against the 3rd scavenger domain of CD163, strongly inhibit Hp-Hb binding (Madsen et al., 2004), but have no detectable effect on bacterial binding as we describe here.

At present we have no information concerning the nature of ligand(s) on bacteria that mediate CD163 recognition. We anticipate that there may be multiple (polyanionic) ligands associated with bacteria. This is firstly based on the observation that CD163 mediates recognition of various bacterial species, including both gram-positive and gram-negative ones that have a rather different outer molecular surface. Indeed, some of the SRCR superfamily members are quite promiscuous. Gp-340 and MARCO, for instance, are able bind to polyanionic bacterial cell wall components, such as LTA and LPS (Elomaa et al., 1998; Brannstrom et al., 2002).

Although our results provide evidence for the recognition of bacteria by CD163 our preliminary experiments have not been able to demonstrate a significant role in bacterial phagocytosis. In apparent contrast, there is good evidence for the uptake, by endocytosis, of Hp-Hb complexes (Nielsen et al., 2006). At present we do not have a satisfactory explanation for this discrepancy. One possibility is that CD163 ligation does not trigger the specific cytoskeletal rearrangements required for phagocytosis.

Our finding that bacterial recognition by CD163 results in a potent inflammatory cytokine response identifies CD163 as a putative sensor for bacterial infection capable of initiating an "early induced" response (see also Janeway et al.) that will likewise contribute to the recruitment of leukocytes and activation of immune effector functions. Clearly, the broad expression of CD163 among macrophages is in line with this. CD163 is highly expressed on most subsets of mature tissue macrophages in the body, including those found in e.g. skin, mucosa, liver and spleen (Van den Heuvel et al., 1999) (Chapter 8). Interestingly, some macrophage subsets such as alveolar macrophages in the lung and macrophages in the splenic marginal zone specifically lack CD163 expression. This may help to prevent excessive local inflammatory

responses as a result of normal exposure in the lung, or that of systemic inflammation in the case of blood-borne bacterial infections in the spleen.

Clearly, the availability of antagonistic antibodies against CD163 offers therapeutic potential for limiting excessive inflammatory cytokine production during severe conditions of infection, such as sepsis. The potent suppressive effect of these antibodies on cytokine production, combined with their minimal effects on bacterial binding and/or phagocytosis makes them, in theory at least, suitable for therapeutic intervention during infection.

Taken together, our findings provide evidence for a novel role of CD163 as a pattern recognition receptor that mediates the recognition of bacteria by macrophages and the triggering of proinflammatory cytokine production. This suggests a contribution of CD163 in the host defense against bacterial infection. The availability of CD163-deficient mice will be helpful for analyzing the actual contribution of CD163 to systemic and local inflammatory responses *in vivo*.

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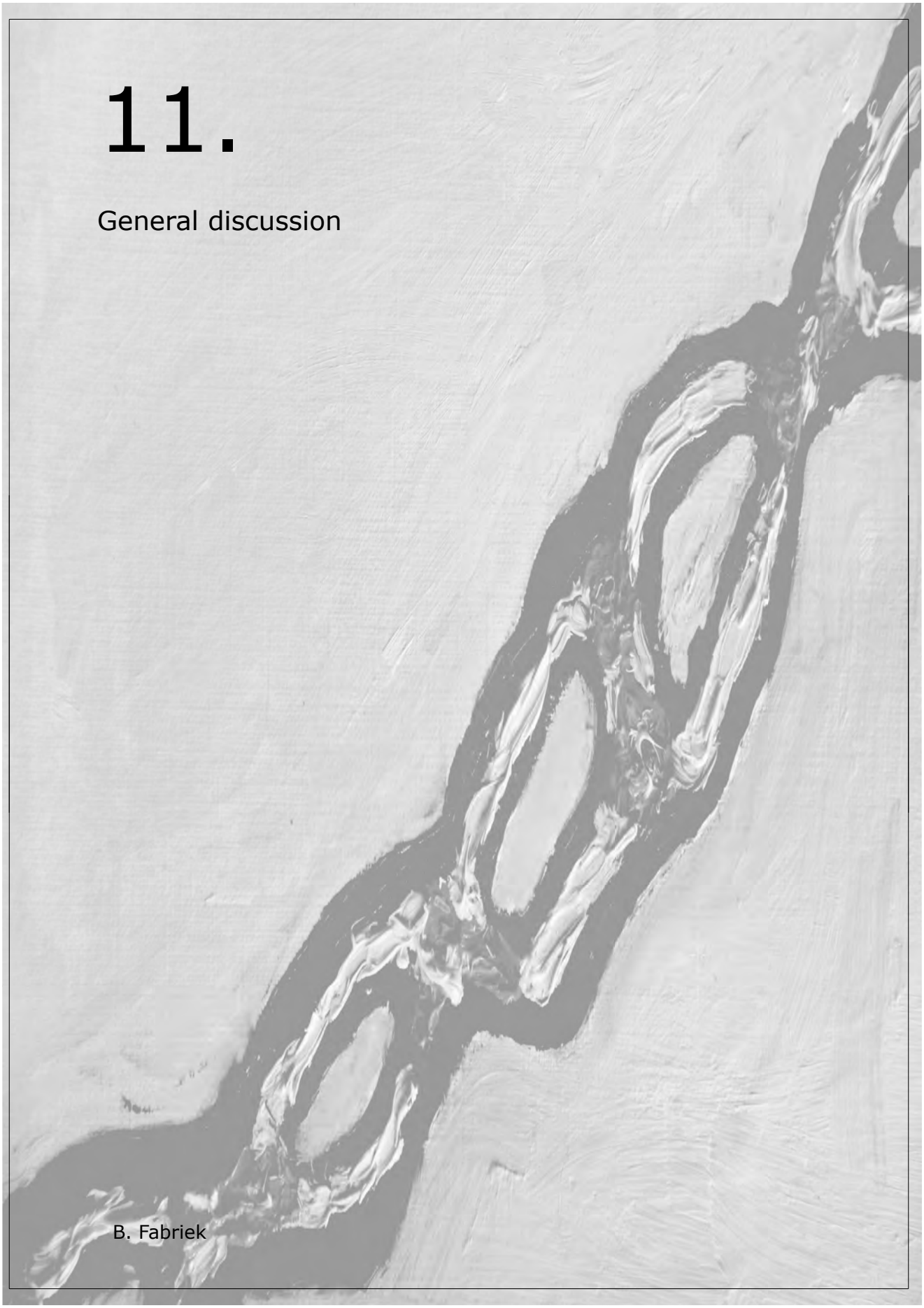
11.

General discussion



B. Fabriek

Chapter 11: General Discussion

Studies described in this thesis were aimed to gain more insight in the role of the perivascular macrophage (PVM) in the brain during neuro-inflammation and in particular with respect to the role of the scavenger receptor CD163 that is expressed by PVM. Many aspects of the findings reported in this thesis have already been discussed in previous chapters. In this general discussion we would like to focus on some of these results, place these findings in a broader perspective, and suggest future lines of research.

PVM are strategically located within the blood-brain barrier (BBB) and are likely to have important functions in both health and disease. The BBB is a structure composed of several different cell types, including endothelial cells, PVM, astrocytes, and their extracellular matrix. The perivascular space, between the endothelial cells and the astrocytes, represents the microenvironment in which the PVM is situated. PVM are most likely responsible for the recognition and uptake of pathogens and their degradation products from the circulation and brain parenchyma and thereby play a pivotal role as scavengers of the CNS, as well as a role in the regulation and development of neuro-inflammatory processes.

Whether the PVM is critical for the initiation of immunity in the CNS remains to be established. Studies in our laboratory provided evidence for an immunoregulatory role of these cells. In particular, the selective elimination of PVM using clodronate liposomes demonstrated an essential role for PVM in meningitis (Polfliet et al., 2001a). PVM were not only shown to play a critical role in bacterial clearance during meningitis, but the results also suggested a facilitating role in leukocyte (i.e. granulocyte) influx into the CNS. Moreover, using the same liposome-based PVM depletion method evidence was also obtained for a supportive role for PVM in experimental autoimmune encephalomyelitis (EAE) (Polfliet et al., 2002). While it was clear that the PVM is not absolutely required for the development of EAE, their elimination lead to a significant decrease in clinical symptoms. The latter was again consistent with a possible role in leukocyte influx, in this case of monocytes and lymphocytes (Polfliet et al., 2001a; Polfliet et al., 2002). While these observations provide critical evidence that PVM represent an important player during immunological processes in the CNS, they did not provide insight into the underlying mechanisms. This is of particular relevance since PVM are at an attractive perivascular location for therapeutic intervention in neuro-inflammatory diseases.

It seems clear that PVM are capable to respond to inflammatory stimuli. They express a variety of pathogen-associated molecular pattern (PAMP) receptors (discussed in paragraph 2), as well as the type 1 interleukin (IL)-1 receptor (Schiltz and Sawchenko, 2002). Once stimulated, they can secrete IL-1β (Van Dam et al., 1992; Bauer et al., 1993; Angelov et al., 1998a) and tumor necrosis factor (TNF)-α (Mato et al., 1998) and express immunocytochemically detectable amounts of cyclo-oxygenase-2 (Elmquist et al., 1997; Schiltz and Sawchenko, 2002) and inducible nitric oxide (NO) synthase (Mato et al., 1998).

Several tools have been used to examine the function of the PVM, including bone marrow chimerism, and clodronate liposomes (Hickey and Kimura, 1988; De Groot et al., 1992; Krall et al., 1994; Priller et al., 2001; Polfliet et al., 2001b; Greter et al., 2005) and in this way scavenger, chemotactic, and antigen presentation functions of PVM have been probed. However, data obtained are usually not conclusive for specific PVM function, since other cell types in the brain or peripheral immune system are also affected. Identification of specific markers for PVM and the developing new methods to study their functional role in *in vivo* animal models might open new perspectives for research on these cells.

1. Markers for brain PVM

Combined myeloid marker detection has been used to distinguish PVM from microglia and pericytes in humans. However, no single marker was exclusive for PVM (reviewed in Chapter 2, paragraph 1B). In rodents the macrophage associated antigen ED2, later identified by us as CD163 (Chapter 9), selectively identifies PVM and meningeal macrophages (MM) in control and inflamed brains (Graeber et al., 1989; Polfliet et al., 2002). In humans we showed that the scavenger receptor CD163 is also an exclusive marker for PVM and MM (Van den Heuvel et al., 1999; Kim et al., 2006)(Chapter 4) in the non-inflamed brain. However, under inflammatory conditions, like MS, foamy macrophages containing myelin debris in active MS lesions were also weakly CD163-positive (Boven et al., 2006) (Chapter 4). In human, 10-30% of the blood monocytes are CD163-positive (Hogger et al., 1998; Van den Heuvel et al., 1999; Sulahian et al., 2000), although direct proof is essentially lacking, we believe that foamy macrophages are probably in large part derived from monocyte that have infiltrated the CNS during MS.

In any case CD163 is apparently not a selective marker for PVM during neuro-inflammation in humans. During our studies we came to the conclusion that the mannose receptor (MR) would be more suitable for selective detection of PVM in MS, since the MR exclusively stains PVM in control as well as in inflamed human brain (Chapter 4). Another advantage of the MR as a marker for PVM is that it is also applicable in experimental studies in mice, which is the most suitable organism for genetic manipulation or deletion of specific genes. In fact, the MR has previously been described to be a good marker for PVM in mice (Galea et al., 2005). CD163 has only briefly been described in the mouse brain and requires more elaborate studies to conclude on its value as a cellular marker for PVM (Kim et al., 2006). At least in humans and mice the MR seems to be a more suitable marker to study PVM in inflammatory conditions than CD163. To our knowledge studies on MR expression in the brain of rats and primates are still lacking. In primates, CD14 is exclusively expressed by PVM and is not found on microglia. CD14 expression has been used to distinguish between these two populations in simian immunodeficiency virus encephalitis (Williams et al., 2001). However, a recent report also suggests that CD163 may perhaps also constitute a selective marker for PVM in primates (Kim et al., 2006).

Apart from their location, morphology and expression of surface markers, the phagocytic capacity of PVM has been used as a marker since PVM are the predominant

phagocytic cells in the CNS, certainly in the absence of inflammation (Kida et al., 1993; Angelov et al., 1998b; Walther et al., 2001; Polfliet et al., 2001b). This may circumvent the possibility that not all PVM express markers such as CD163, which might result in an underestimation of their number. However, a recently published study by Kim and colleagues demonstrates that after dextran-dye labeling all phagocytosing cells were indeed CD163-positive in primates (Kim et al., 2006). However, there are of course major limitations for studying phagocytosis in human subjects.

We have shown for the first time that different subpopulations of PVM expressing different receptors and with a different morphology can be distinguished (Chapter 4). One might speculate that these subpopulations represent different maturation stages of PVM and/or reflect a certain level of functional specialization of the PVM. A number of studies have been reported in the last years which point to a specific population of CD11c-positive dendritic cells (Matyszak and Perry, 1996; Greter et al., 2005), that are located in the meninges, choroid plexus, and perivascular space. At present we do not know whether these populations overlap in some way with the PVM population(s) that we have been studying. Clearly, further studies should be performed to clarify this and further characterize these subpopulations with possible differences in morphology, phenotype, and function. Overall, we believe that the PVM plays a very important role in various processes in the brain, such as maintenance of the BBB, pathogen clearance, antigen presentation and many more (an overview is given in Figure 1).

2. Do PVM function as antigen presenting cells in the brain?

Until recently the general idea was that the brain lacks typical antigen presenting cells (APC). However, as indicated above, several studies have suggested that there may actually be APC present in the CNS and the PVM is clearly a potential candidate (Hickey and Kimura, 1988; Ulvestad et al., 1994; Matyszak and Perry, 1996; Angelov et al., 1996; Angelov et al., 1998b; Polfliet et al., 2002; Suter et al., 2003). Several features of PVM are consistent with a putative role as APC of the brain, including their strategic location at the BBB, their bone marrow origin, a prominent role in antigen/pathogen scavenging (by virtue of the various endocytic/phagocytic receptors including the MR, DC-SIGN and CD163 (Chapter 4), and their constitutive MHC class II expression (reviewed in Chapter 2 and summarized in Figure 1). Microglial cells and astrocytes in the CNS parenchyma lack many of the molecules relevant for antigen presentation, including detectable levels of surface MHC class I and II, at least under normal conditions (Hickey and Kimura, 1988; Streit et al., 1989; Antel and Owens, 1999). Thus, although not directly demonstrated, PVM are perhaps the prime candidates to act as local APC in the brain. As a result they may be critical for the initiation of immunity in the CNS and, as such, play an important role in various brain disorders, including MS.

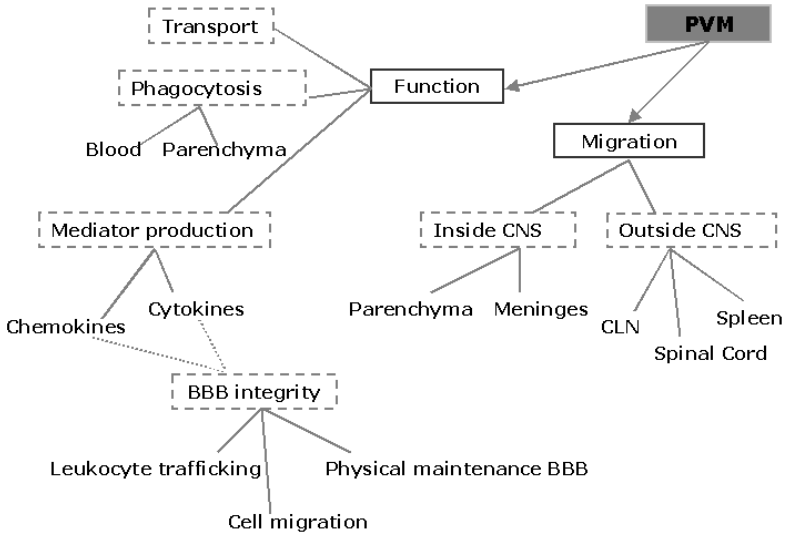


***Figure 1**: Schematic overview of PVM functions in the brain.*

Due to the strategic location of the PVM at the BBB, there is evidence for a wide range of functions, in which the PVM is involved, in this schematic overview a selection is depicted.

3. A role for the scavenger receptor CD163 in inflammation

In order to gain insight into the functions of PVM during inflammation in general and in the CNS in particular, we focussed on the scavenger receptor CD163 which is, as indicated previously, expressed on PVM in the CNS of rodents (Graeber et al., 1989), primates (Kim et al., 2006), and humans (Van den Heuvel et al., 1999; Kim et al., 2006) (Chapter 4). CD163 in human is expressed on most subpopulations of mature tissue macrophages, including splenic red pulp macrophages, resident bone marrow macrophages, and Kupffer cells of the liver (Van den Heuvel et al., 1999). There is also a low expression level on monocytes. In rodents similar expression patterns can be observed for the ED2 antigen, which is the rat ortholog of CD163 (Chapter 9), with the notable difference that CD163 expression is not found on monocytes (Chapter 8). It is anticipated that CD163 is not only involved in CNS-related inflammatory processes but rather plays general functions in homeostasis and/or host defense (reviewed in Chapter 3).

3.1 Novel functions for CD163 in cell-cell adhesion and host defense.

The first indication for a potential regulatory role of CD163 in inflammation was our previous finding that *in vitro* cross-linking of human CD163 on the cell surface with EDHu-1, an anti-CD163 antibody, induces the secretion of cytokines in monocytes, such as IL-1β, IL-6, IL-10, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Van den Heuvel et al., 1999). Our findings in Chapter 8 suggest that the capacity to trigger inflammatory mediators is shared by rat CD163, which upon ligation triggers NO, TNF-, IL-1β and IL-6 production by macrophages. Although the underlying signalling pathway is only poorly understood, there is evidence supporting an involvement for protein tyrosine kinase, protein kinase C, and casein kinase II (Van den Heuvel et al., 1999; Ritter et al., 2001). Of course, an important question in this context, which is for instance addressed in Chapters 9 and 10, is what the natural ligands for CD163 are and whether these would be able to trigger cytokine production.

Thus far, the best characterized function for CD163 remains that it has been identified as a scavenger receptor for hemoglobin-haptoglobin (Hb-Hp) complexes (but not free Hb) (Kristiansen et al., 2001; Madsen et al., 2001; Graversen et al., 2002). Free Hb is a strong oxidant and can therefore be toxic to the host, particularly during hemolytic conditions, such as autoimmune hemolytic anemia, sickle cell disease or malaria (Wagener et al., 2003). CD163 is believed to play a substantial role in the clearance of free Hb, in part through the clearance of Hb and its effects on anti-inflammatory pathways through IL-10 and heme-oxygenase production (Philippidis et al., 2004). Mutagenesis and monoclonal antibody inhibition studies have suggested that the third SRCR domain is most likely to harbor the binding site for Hb-Hp (Madsen et al., 2004).

One of the subpopulations of macrophages that express high levels of CD163 are resident bone marrow macrophages. These cells together with developing erythroid cells form the so-called erythroblastic islands. The intimate association between macrophages and erythroblasts is believed to be essential for erythropoiesis. Our studies identify a potential role for CD163 in this process. In particular, we demonstrate that CD163 functions as a receptor for immature erythroblasts and interactions with CD163 promote the proliferation and/or survival of developing erythroblasts (Chapter 9). The potential cell-cell interaction site was identified in the second SRCR domain of CD163.

Of interest, although we have no solid evidence to support this yet, CD163 might mediate cell-cell interactions with other hematopoietic cells, which could be relevant during inflammation. This is supported by the previously reported interaction of soluble CD163 with T lymphocytes (Hogger and Sorg, 2001; Frings et al., 2002). It will be interesting to investigate this further and to test whether putative interactions between macrophages and other cells will regulate CD163-mediated cytokine production.

Our studies identify CD163 as a novel pattern recognition receptor for Gram-positive and Gram-negative bacteria (Chapter 10). Other members of the SRCR family, including the soluble molecules gp-340 and Spα, and the macrophage receptor MARCO, are also involved in the recognition of bacteria and it seems possible that bacterial recognition constitutes a common and ancient property of SRCR family members.

Importantly, we have demonstrated that bacterial recognition by CD163 triggers a potent cytokine response. The latter suggests that CD163, as a molecule broadly expressed on macrophages throughout the body, may act as a typical innate sensor for bacterial infection and an inducer of a so-called early induced response (Janeway, Jr., 1992). Our findings do not only provide direct evidence for a role of CD163 in the host response against infection, but may perhaps also be exploited for therapeutic purposes. In particular during severe bacterial infections, such as sepsis, the application of antagonistic antibodies against CD163 that we have generated may result in beneficial effects.

The meningeal macrophages, which we consider as a subset of PVM, express high levels of CD163 in both humans and rodents. We have previously demonstrated that meningeal macrophages play a critical and protective role in the development of bacterial meningitis (Polfliet et al., 2001a). The results suggested that meningeal macrophages might not only play a primary role in the clearance of bacteria, but also act by facilitating granulocyte influx into the meninges, which are believed to play a dominant role in pathogen removal. Clearly, a role of CD163 as a sensor for bacteria and an inducer of other inflammatory effector mechanisms as indicated above are in line with this.

Taken together, our findings as well as those of others picture CD163 as a typical macrophage scavenger receptor that mediates interactions with a structurally diverse set of ligands, including Hb-Hp as well as putative molecules on host cells, and PAMPs on pathogens. These interactions may play a homeostatic role related to the biology of red blood cells, by supporting erythroid development and the clearance of Hb. On the other hand, the recognition of bacteria, but perhaps also that of Hb-Hp complexes, may lead to cytokine production, and this is anticipated to be important for the host defense against infection.

3.2 Regulation of CD163 expression

Another potentially relevant link between CD163 and inflammation is the well-documented regulation of CD163 by glucocorticoids (GC) and cytokines. The prevailing picture is that CD163 is upregulated by anti-inflammatory agents (GC and anti-inflammatory cytokines) and can be downregulated by pro-inflammatory agents (Buechler et al., 2000). However, the role of these agents in regulating the expression of CD163 *in vivo* had until now not been established. Our work (Chapter 7) shows CD163 upregulation during high dose GC treatment in MS patients. The results also demonstrate that the *in vitro* inducibility on monocytes holds some promise for predicting GC responsiveness in MS, and perhaps also in other relevant diseases, such as allergy or rheumatoid arthritis (RA). This provides the first evidence for regulation of CD163 by GC *in vivo*.

The upregulation of CD163 by GC may have implications in the context of bacterial infections. GC are frequently employed for treating chronic inflammatory conditions, such as MS, RA and allergies. One of the major effects of GC is a suppression of pro-inflammatory cytokine production by macrophages and other cells. In spite of well established inhibitory effects of GC on LPS- or lipoteichoic acid-induced (i.e. Toll-like

receptor-mediated) cytokine production and the sometimes devastating effects of cytokines during severe bacterial infection, GC treatment does not appear to have beneficial effects during such conditions (Annane, 2005). Our current findings suggest that the known suppressive effects of GC on TLR-mediated cytokine production may be balanced by an upregulation of CD163, which is anticipated to sensitize cytokine production by intact bacteria or other PAMPs. One potential solution could be to combine GC treatment with the CD163 antagonists that we have generated in Chapter 10.

CD163 has been indicated as a typical marker for so-called alternatively activated macrophages or M2 macrophages. The counterpart of the M2 macrophage is classically activated (M1), which is obtained by stimulation with interferon (IFN)-γ, tumor necrosis factor (TNF)-α or lipopolysaccharide (LPS), and is characterized by a high production of pro-inflammatory mediators. On the other hand, the M2 macrophage can be induced by IL-4, IL-10, or GC and is associated with tissue remodeling, tissue repair, and debris scavenging (Mantovani et al., 2002; Gordon, 2003; Mosser, 2003). Although the classification of macrophages within the above-mentioned subtypes facilitates a phenotypical distinction, the assumption on differences in functional capacities of these two different macrophage subpopulations can be misleading. In fact, our present functional experiments argue in favor of a possible causal link between CD163 and a pro-inflammatory, classically activated macrophage phenotype, whereas on the basis of receptor expression this macrophage would rather be designated as an alternative activated phenotype. The fact is that CD163 is expressed on most subsets of macrophages in the body suggesting that it is rather a marker for most mature tissue macrophage subsets (Chapter 8).

In addition to membrane bound CD163 present on monocytes and macrophages, a soluble form of CD163 (sCD163) has been found in plasma and other tissue fluids (Moller et al., 2002). CD163 can be shed from the cell membrane by a TIMP-3-sensitive metalloproteinase in response to LPS and phorbol esters *in vitro* (Droste et al., 1999; Hintz et al., 2002; Timmermann and Hogger, 2005). Moreover, recent studies have shown that CD163 can also be released from monocytes in response to selective activation of TLR 2, 4, and 5 as an acute innate response to extracellular pathogen infections (Weaver et al., 2006). The latter may be of relevance during bacterial infection, where proteolytic cleavage may act as a feedback mechanism to prevent excessive inflammatory cytokine production. The soluble molecule is slightly smaller than the membrane bound protein indicating that the proteolytic cleavage site is probably localized near the cell membrane (Moller et al., 2002) and cleavage will most probably remove the ligand binding sites in SRCR domains 2 and 3.

Whereas an increase in membrane CD163 expression is only reported in a small number of diseases, increased sCD163 levels have been found elevated in a wide range of pathologies (Moestrup and Moller, 2004) all having one feature in common, sCD163 reflects macrophage function and monocyte/macrophage load in the body. In MS we observed increased levels of sCD163 related to a decrease in monocyte surface CD163 expression, which is consistent with an increased level of proteolytic shedding (Chapter 6) (Droste et al., 1999; Hintz et al., 2002; Timmermann and Hogger, 2005). Our

results also provide direct evidence for the existence of a CD163 cleaving MMP-like protease activity in the plasma of MS patients. The levels of this activity correlated with the amounts of sCD163 in plasma. If the triggering of CD163, by for instance cells (Chapter 9), bacteria (Chapter 10), Hp-Hb complexes (Kristiansen et al., 2001), and/or other unidentified ligands, does indeed contribute to the local and/or systemic production of inflammatory cytokines, then the proteolytic shedding may be a way to provide negative feedback to prevent excessive cytokine production. Indeed, the negative relationship between CD163 shedding and circulating cytokine levels (Chapter 6) provides indirect support for this idea.

Soluble receptors, such as soluble MR and α_2-macroglobulin, suggests that these soluble receptors might function as antigen-scavengers either for cross-linking of the receptor on the cell, or for transport of the antigen towards other sites of the body or other cell-types for clearance, and in this way prevent receptor triggering (Moestrup and Gliemann, 1991; Martinez-Pomares et al., 1998; Jordens et al., 1999). However, no such function for sCD163 has been described or studied up to now. Whether the sCD163 molecule itself has any physiological function is not really known. Some studies postulate a potential anti-inflammatory function for sCD163, since it decreases T lymphocyte activation and proliferation *in vitro* (Hogger and Sorg, 2001; Frings et al., 2002), however, this should be investigated in more detail.

Taken together, several possible pro- and anti-inflammatory functions have been postulated for CD163 mainly based on findings from *in vitro* studies or *in vivo* observations. However, the precise contribution of CD163 in inflammatory processes maybe best studied by *in vivo* experiments where CD163 is selectively blocked or absent (e.g. CD163 knock out mice) during inflammation.

4. Concluding remarks

CD163 upregulation on PVM precedes the onset of clinical symptoms and leukocyte infiltration in EAE (Polfliet et al., 2002). These observations support the idea that the PVM strategically located at the BBB act as a sensor for the CNS inflammatory response in the early stages of inflammation. It will be of interest to identify the cellular ligand of CD163 and investigate whether this is present on other cell types. Whether this will lead to the production of pro-inflammatory cytokines *in vivo*, and contribute to leukocyte infiltration into the CNS during inflammation is also a subject for future studies.

Evidence in this thesis supports the idea that during the initial phase of bacterial meningitis, the PVM and MM contribute to the recognition and phagocytosis of bacteria via CD163 present on these cells. Subsequently, pro-inflammatory cytokines are released that in turn mediate cell attraction and BBB permeability. Data supporting this hypothesis come from studies where the PVM and MM are selectively depleted and meningitis is induced. Depleted animals show a reduced influx in granulocytes, which fully supports our hypothesis (Polfliet et al., 2001a). Future studies should address the question if CD163 is indeed involved in the *in vivo* recognition and phagocytosis of bacteria.

We believe that the function of PVM in the brain is largely based on their surface molecule expression. There has been a significant advance in understanding the fundamental properties of the scavenger receptor CD163 in homeostasis and host defense. Whether CD163 also constitutes a suitable target for reducing cytokine production during infection or chronic inflammatory conditions, such as MS, remains to be established. The best way to functionally study the role of the perivascular-macrophage associated receptor CD163 in neuro-inflammation would be to study meningitis or EAE in CD163 knock-out animals.

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Summary

The brain is protected from invaders by the presence of an endothelial blood-brain barrier (BBB). This implies that the bloodvessels of the brain are different from the rest of the body; they are less permeable for pathogens and large proteins by the presence of tight junctions between endothelial cells surrounding these vessels. Around these bloodvessels perivascular macrophages (PVM) are located. PVM might play a role in the recognition and uptake of pathogens and their degradation products present in the bloodstream or from the brain parenchyma (Kida et al., 1993; Mato et al., 1996), and as such may contribute significantly to the initiation of both innate as well as adaptive immune responses in the brain. The PVM express a variety of receptors enabling pathogen recognition and uptake, including members of the scavenger receptor family. These receptors have a relatively broad ligand binding specificity (Resnick et al., 1994; Krieger, 1997) and can mediate the recognition and uptake of a variety of pathogens, including viruses, bacteria and fungi. The aim of this thesis was to investigate the role of the PVM and the PVM-associated scavenger receptor CD163 in the development of CNS inflammation.

Functional evidence suggests that PVM play a supportive role during experimental autoimmune encephalomyelitis (EAE) in rodents (Polfliet et al., 2002), an animal model for Multiple Sclerosis (MS). However, PVM in the human CNS were still poorly characterized. We investigated CD163 expression in the normal human brain and in the brain of MS patients (Chapter 4) and showed expression of CD163, primarily on PVM and to a lesser extent on myelin-containing 'foamy' macrophages within MS lesions. To gain more insight into the function of PVM in antigen recognition and presentation we studied the co-expression of DC-SIGN, mannose receptor, MHC class II, and several costimulatory molecules by PVM in the normal and inflamed human CNS (MS brain lesions). A subpopulation of the CD163-positive PVM in the human brain express several molecules involved in antigen recognition, presentation, and costimulation. Therefore PVM are equipped to recognize antigen and present it to T cells, supporting a role in the regulation of perivascular inflammation in the human CNS.

The cervical lymph nodes (CLN) are the first draining site of the brain and therefore possibly reflect the first site of encounter between myelin antigens and naïve T lymphocytes (Weller, 1998). Non-human primates with EAE showed the presence of myelin components in cells expressing dendritic cell and macrophage markers in the CLN (De Vos et al., 2002). By means of ultrasound guided fine needle aspiration cytology (USgFNAC) we obtained cells, *in vivo*, from non-enlarged CLN of MS patients and healthy controls (HC), and found macrophages containing myelin proteins in the CLN of all MS patients, whereas these could only be detected in a minority of HC (Chapter 5). These findings are consistent with antigen transport from the brain to the CLN. Understanding of the mechanisms behind this may be relevant for induction and/or maintenance of autoimmunity in the CNS. Antigen transport in the CNS could either be cell-mediated, cell-independent, or both. PVM are particularly effective scavengers of the perivascular space in the CNS (Kida et al., 1993; Angelov et al.,

1996; Mato et al., 1996) and may thus play a major role in antigen uptake, but the precise route of the ingested particles and antigens remains unclear.

After studying PVM in the human brain and the expression of CD163 on these cells, we investigated the regulation of the scavenger receptor CD163 on monocytes in MS. CD163 is a tissue macrophage marker (Van den Heuvel et al., 1999), but is also expressed on a small percentage of blood monocytes in humans. CD163 has been implicated to play a role in both homeostatic and inflammatory processes (Moestrup and Moller, 2004) and proteolytic shedding of CD163 from the cell membrane results in a soluble form of the receptor, sCD163 (Droste et al., 1999; Moller et al., 2002; Timmermann and Hogger, 2005). Increased sCD163 levels have been found elevated in a wide range of pathologies (Moestrup and Moller, 2004). In MS, plasma sCD163 was found increased and monocyte membrane CD163 decreased (Chapter 6). sCD163 levels appear dependent not only on the surface expression of CD163, but also on the presence of proteases and protease inhibitors that regulate CD163 shedding (Chapter 6) (Droste et al., 1999; Hintz et al., 2002; Timmermann and Hogger, 2005).

As indicated above CD163 can be shed from the cell membrane by metalloproteinase activity in response to LPS and phorbol esters *in vitro* (Droste et al., 1999; Hintz et al., 2002; Timmermann and Hogger, 2005). However, CD163 can be potently upregulated by glucocorticoids (GC) *in vitro* (Chapter 9) (Hogger et al., 1998; Van den Heuvel et al., 1999) and *in vivo* (Chapter 7). Although a higher production of CD163 can be found in response to GC *in vivo*, this does not hold true for sCD163 levels after GC. Moreover, we provided evidence that measuring membrane CD163 response *in vitro* might have prognostic value for predicting individual *in vivo* glucocorticoid responsiveness of MS patients.

Although a role for CD163 in inflammatory diseases has been postulated the only two well described functions of CD163 are its binding to hemoglobin (Hb)-haptoglobin (Hp) complexes resulting in endocytosis (Kristiansen et al., 2001; Graversen et al., 2002; Madsen et al., 2004) and the capacity of CD163 to trigger cytokine production (Van den Heuvel et al., 1999). In this thesis we identified the rat macrophage ED2 surface antigen as the ortolog of human CD163 (Chapter 9). Moreover, triggering of rat CD163 was shown to induce cytokine and nitric oxide production in macrophages (Chapter 8). We also studied other potential functional aspects of the CD163 molecule in more detail. In particular, our findings implicate CD163 on macrophages as a receptor for erythroblast and suggest a regulatory role for CD163 in erythropoiesis (Chapter 9). This is the first demonstration of a role for CD163 in cell-cell interactions between macrophages and hematopoietic cells and this could also be relevant during (CNS) inflammation.

Two secreted molecules of the scavenger receptor cysteine-rich superfamily class B (SRCR-B), i.e. the salivary agglutinin gp-340 and Spα, have been shown to bind Gram-positive and Gram-negative bacteria (Sarrias et al., 2005). We demonstrated that CD163, also a member of the SRCR-B family, can mediate bacterial recognition and that the second SRCR domain of the molecule mediates this. Furthermore, we demonstrate that bacterial recognition triggers CD163-dependent cytokine production by macrophages. This demonstrates for the first time that CD163 on macrophages can

act as a microbial receptor and suggest a role as an innate sensor in the host response against bacterial infection (Chapter 10).

Collectively, these observations support the idea that the PVM strategically located at the BBB acts as a sensor during inflammatory responses within or outside the CNS. CD163 may be one of the important receptors on PVM by which it may sense inflammation or infection. We have now established that cellular and bacterial ligands of CD163 result in the production of pro-inflammatory cytokines or other effector functions, which might have implications for the development and pathogenesis of MS and meningitis, whether this will contribute to leukocyte infiltration and bacterial clearance in the CNS during inflammation remains to be established.

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Chapter 4

Chapter 5

Chapter 9

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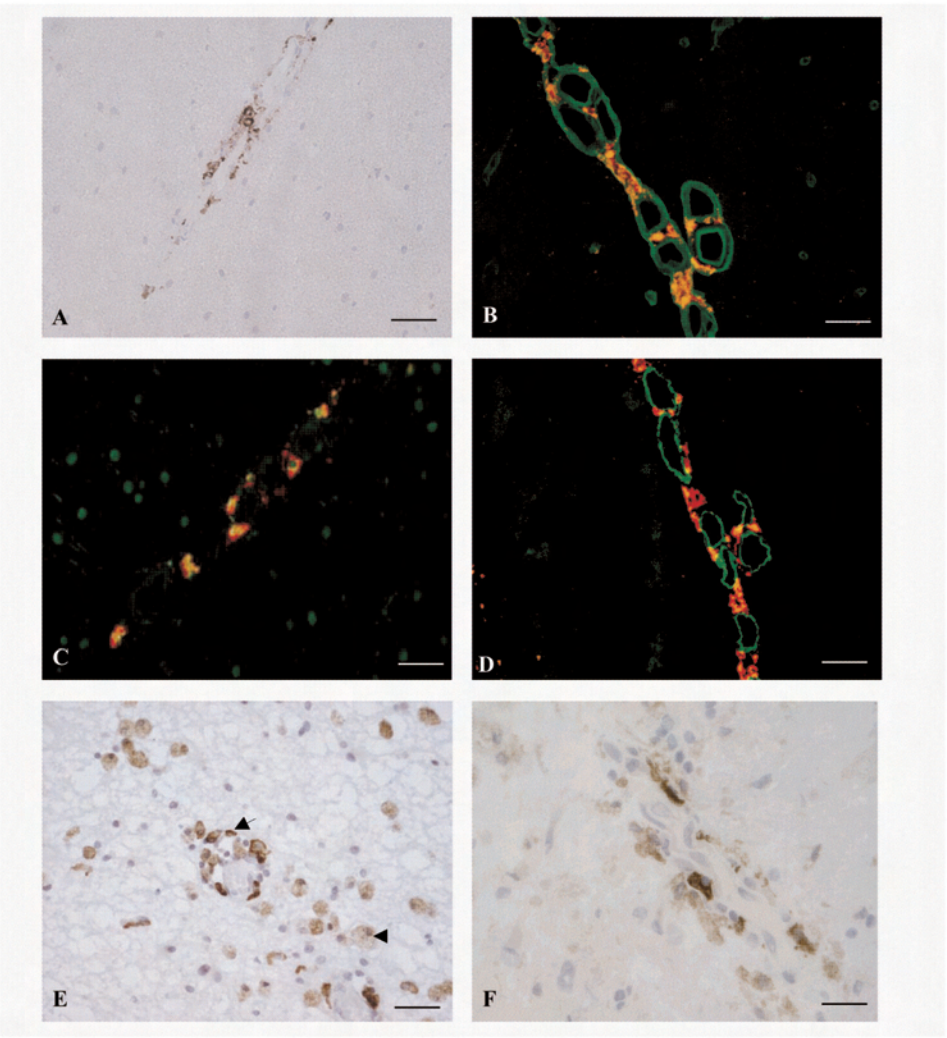

Figure 1: *CD163 expression in normal and MS lesion white matter*

A: Control white matter, frozen section stained with mAb EDHu-1 against CD163. PVM are CD163-positive and have an elongated shape around a medium-sized blood vessel. **B-D:** Control brain, cerebral white matter, frozen section stained with double immunofluorescence for CD163 (red) and laminin (green; **B**), CD68 (green; **C**), smooth muscle actin (green; **D**). CD163-expressing cells are CD68-positive macrophages. The PVM lie between the laminin-positive endothelial and glial basement membranes and are SMA-negative. **E**: Active lesion, frozen section, mAb EDHu-1 against CD163. Parenchymal macrophages (arrowhead, foam cells) are weakly positive. PVM (arrow) near blood vessels at the lesion edge are strongly positive. **F:** Chronic active lesion, paraffin embedded section. On the right the inactive centre of the lesion is seen, with hardly any positive cells. The predominant cell type is the strong staining; rounded PVM, but weaker staining foam cells and some microglial cells are also visible. Scale bar = 100 (A, E) and 50 um (B, C, D, F).

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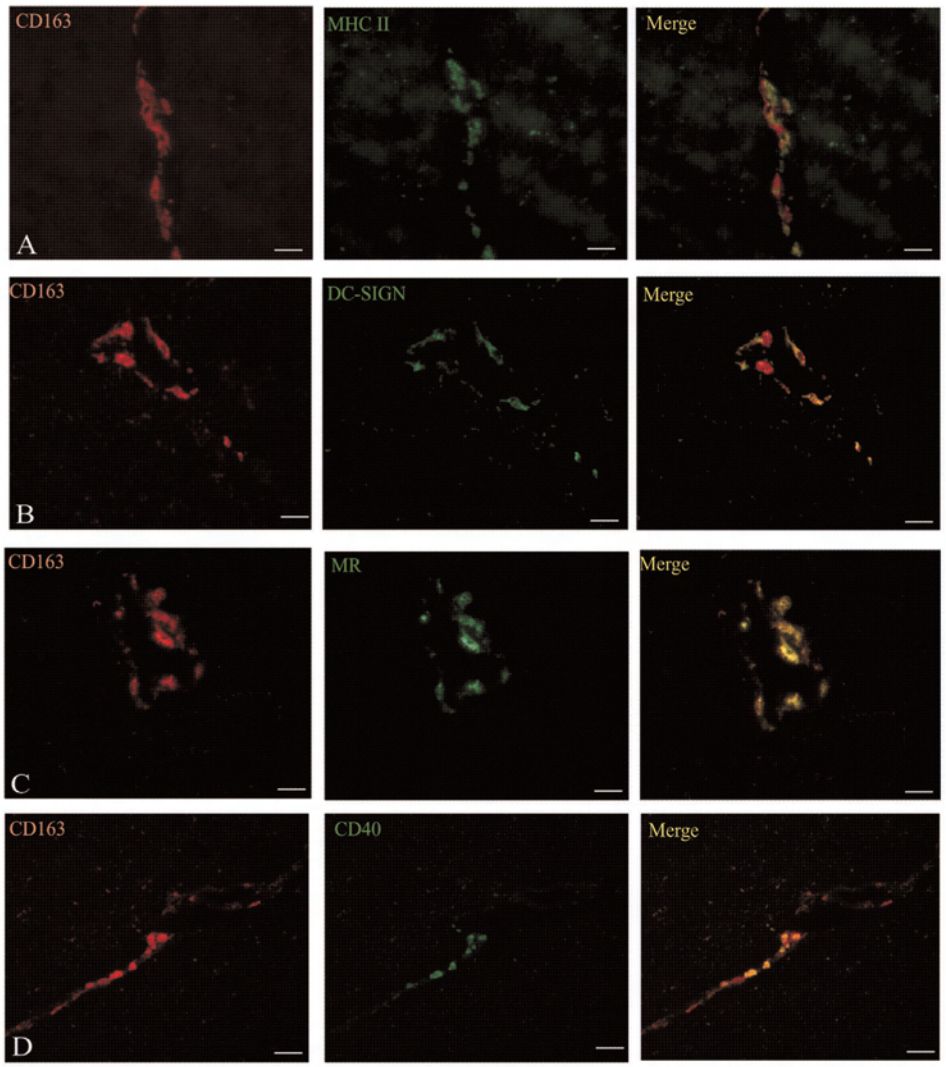

Figure 2: *Double immunofluorescence for CD163 and other molecules in normal white matter.*

A. Control cerebral white matter, double immunofluorescence for MHC class II (green) and CD163 (red). CD163-positive macrophages are positive for MHC class II**. B.** Control cerebral white matter, double immunofluorescence for DC-SIGN (green) and CD163 (red). There is colocalization of CD163 and DC-SIGN on a subpopulation of PVM. **C.** Control cerebral white matter, double immunofluorescence for MR (green) and CD163 (red). All CD163-positive PVM express MR. **D.** Control cerebral white matter, double immunofluorescence for CD40 (green) and CD163 (red). A subpopulation of CD163-positive PVM shows staining for CD40. Scale bar = 50 µm.

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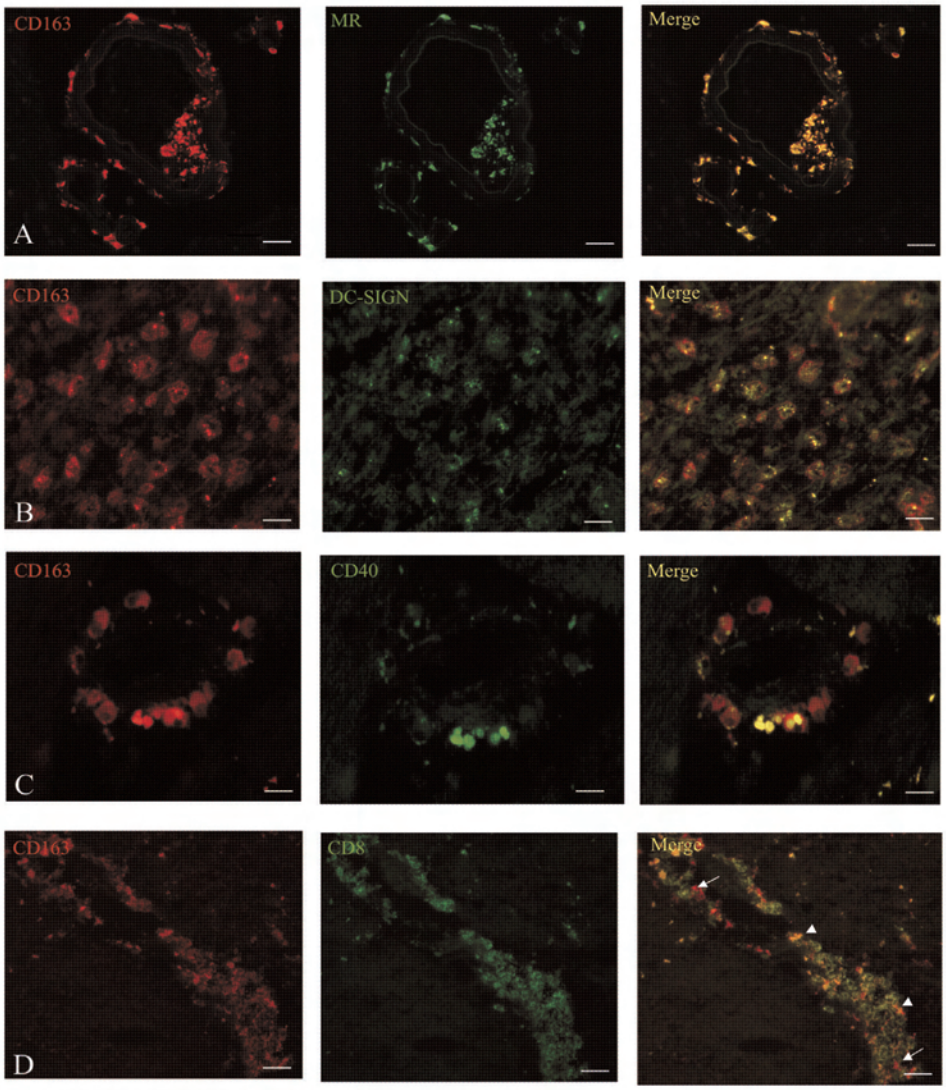

Figure 3: *Double immunofluorescence for CD163 and other molecules in MS lesion white matter.*

A-D; Double immunofluorescence for CD163 and other molecules in MS white matter **A.** Active MS lesion, double immunofluorescence for MR (green) and CD163 (red). Foamy macrophages do not express MR but do express low levels of CD163 (not shown in this picture). **B.** Active MS lesion stained for DC-SIGN (green) and CD163 (red). Foamy macrophages within the lesion are weakly positive for CD163 and also for DC-SIGN. **C.** Active MS lesion, double immunofluorescence for CD40 (green) and CD163 (red). Subpopulations of CD163-positive PVM express CD40. **D.** Chronic active lesion stained for CD8 (green) and CD163 (red). There is close apposition (arrow) of the green lymphocytes with the red PVM and an occasionally overlap (arrowhead) of CD8 and CD163 in an infiltrated vessel near the lesion site. Scale bar = 100 um (A and D), scale bar = 50 µm (B and C).

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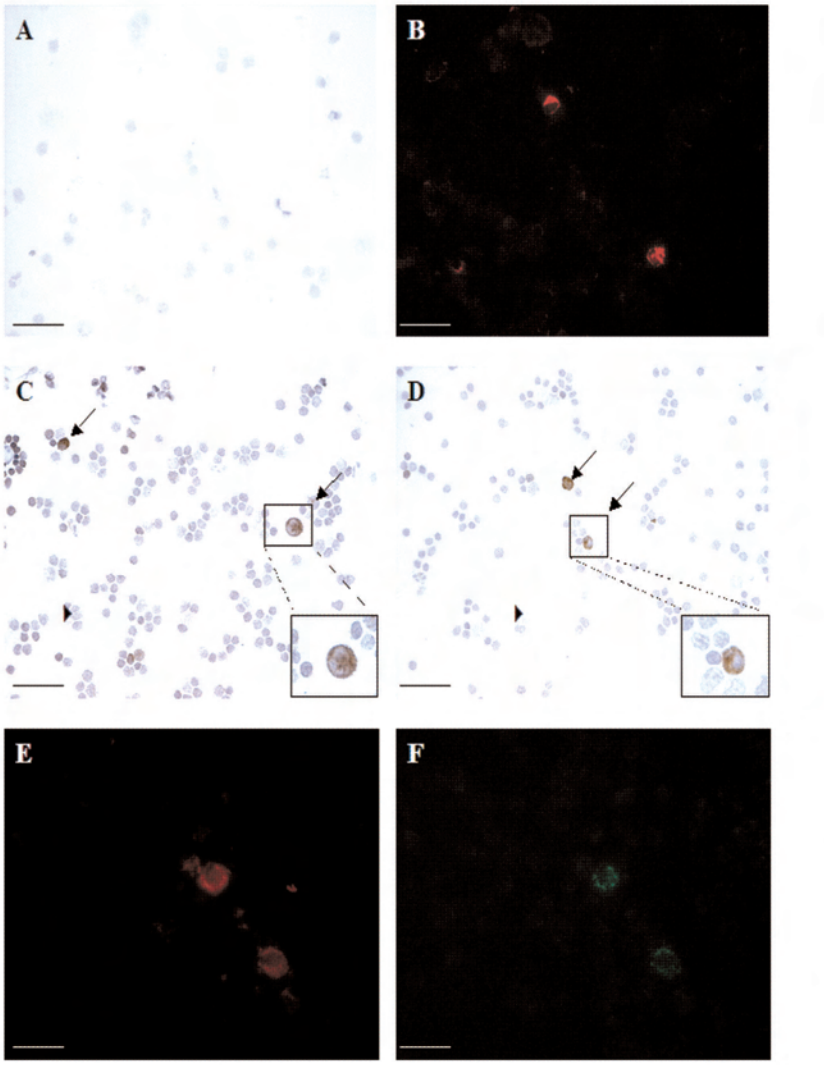

Figure 1: *Immunohistochemical and immunofluorescence staining of CLN aspirates*

A. Immunoperoxidase staining of the CLN material of a HC (nr. 10) stained for MBP. In blue the nuclei of the cells are visible by haematoxylin counterstaining. No MBP staining observed. **B.** CLN material of a healthy control subject (nr. 8) stained for MBP (green) and CD68 (red) by immunofluorescence. Several macrophages (red) are visible whereas no MBP staining (green) can been observed. **C/D.** CLN material of a MS patient (nr. 7) stained by immunoperoxidase for MBP (C) or PLP (D). MBP or PLP positive cells are indicated with an arrow, negative macrophages with an arrowhead**. E/F.** CLN material of an MS patient (nr.7) stained for MBP (green) and CD68 (red) by immunofluorescence. Several macrophages (red) are visible containing MBP (green) (scale bar = 50 µm).

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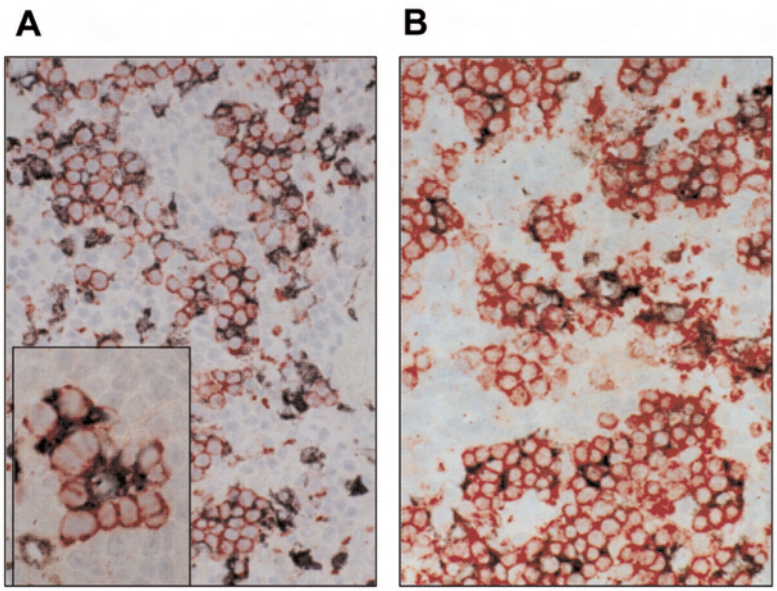
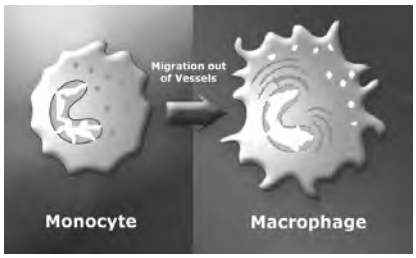


Figure 1: *Expression of the rat ED2 antigen by macrophages in splenic erythroblastic islands.*

Microphotographs from the red pulp areas from the spleens of a juvenile 7 day old rat (**A**), or an adult animal 6 days after *Plasmodium berghei* infection (**B**), double stained with mAb ED2 for rat CD163 (black) and mAb OX2617 for rat transferrin receptor-positive erythroblasts (red).

1. Het immuunsysteem en de macrofaag

Het immuunsysteem beschermt ons lichaam tegen een breed scala aan ziekteverwekkers zoals bacteriën, schimmels, schadelijke stoffen en virussen. Het is in staat om de ziekteverwekkers te herkennen en op te ruimen. Witte bloedcellen maken een onderdeel uit van ons immuunsysteem, een voorbeeld van een witte bloedcel is de monocyt. Monocyten zitten in de bloedbaan. Als ze vanuit het bloed de weefsels in gemigreerd zijn, worden ze macrofagen genoemd (Figuur 1). Macrofagen herkennen ziekteverwekkers, nemen ze op (= fagocytose) en breken ze af. Om ziekteverwekkers te herkennen hebben macrofagen receptoren op het celoppervlak zitten. Deze receptoren herkennen specifieke gedeelten (ligand) van een virus of bacterie dit kan vervolgens een immuunreactie op gang brengen. Een voorbeeld van macrofaagreceptoren is de groep van de scavenger receptoren. Deze macrofaagreceptoren zijn zo genoemd omdat ze afvalstoffen opruimen (scavengen =wegvangen). In dit proefschrift hebben we met name gekeken naar de scavenger receptor CD163. Deze receptor zit op weefselmacrofagen en speelt mogelijk een rol bij ontstekingen in de hersenen, zoals bij de ziektes Multiple Sclerose en hersenvliesontsteking (meningitis).



Figuur 1: *Een schematische weergave van een monocyt en een macrofaag*

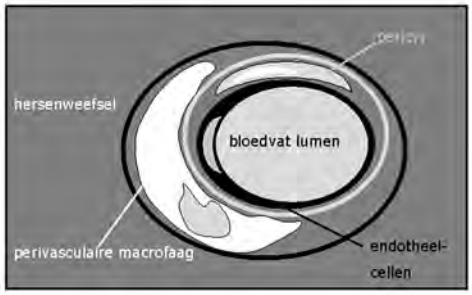
Zodra een monocyt vanuit de bloedbaan naar de weefsels migreert wordt het een macrofaag genoemd. Figuur afkomstig van Graphic and Interactive Design (Brooklyn, MA, USA).

2. Multiple Sclerose

Multiple Sclerose (MS) is een ziekte van het centraal zenuwstelsel (hersenen en ruggenmerg) waarbij neurologische uitvalsverschijnselen optreden, zoals wazig zien, tintelingen in armen en moeite met lopen. De klachten ontstaan doordat de isolatielaag rondom zenuwvezels (myeline) is aangetast, waardoor de geleiding van prikkels is verstoord. MS is een auto-immuunziekte, dat wil zeggen dat het immuunsysteem het lichaam zelf aanvalt. Hierbij komen ontstekingscellen de hersenen binnen en richten daar schade aan.

In de hersenen zijn de bloedvaten anders dan in de rest van het lichaam. Ze zijn minder doorlaatbaar voor ziektekiemen, cellen en grote eiwitten. Dit komt doordat de binnenkant van de bloedvaten bekleed wordt met gespecialiseerde endotheelcellen. Bovendien liggen om de bloedvaten in de hersenen perivasculaire macrofagen (PVM)

(Figuur 2). De PVM beschermen het hersenweefsel tegen binnendringende ziektekiemen en andere ontstekingsverwekkers (poortwachterfunctie). Net als andere weefselmacrofagen in het lichaam zijn is ook PVM in staat om deeltjes in zich op te nemen en af te breken.



Figuur 2: *Schematische weergave van een dwarsdoorsnede door een bloedvat in de hersenen.*

Direct aan het bloedvat lumen ligt een laag met endotheelcellen, deze endotheelcellen liggen zo dicht tegen elkaar aan dat cellen en eiwitten er niet doorheen kunnen. In de buurt van deze endotheelcellen liggen de pericyten welke ervoor zorgen dat het bloedvat elastisch is. In een ander compartiment liggen de PVM.

3. Promotieonderzoek

Het doel van dit promotieonderzoek was om de rol van PVM tijdens ontsteking in de hersenen te bepalen en specifieker de rol van de macrofaagreceptor CD163 bij dit proces.

3.1. De perivasculaire macrofaag in de hersenen

De PVM liggen op een strategische plaats in de hersenen, namelijk tussen de periferie (de bloedbaan) en het hersenweefsel (zie Figuur 2). Door deze strategische ligging kan deze cel relatief gemakkelijk een immuunreactie in het hersenen op gang brengen. Of deze cel daartoe in staat is, was nog onbekend (Hoofdstuk 4). We onderzochten of de scavenger receptor CD163 op PVM zit en bekeken de aanwezigheid van dit molecuul in verschillende MS-laesies (ontstekingshaarden) in de hersenen.

In MS-laesies is er meer CD163 te vinden dan in gezond hersenweefsel en brengen macrofagen, die vol met myeline afbraakproducten zitten, CD163 tot expressie. Naast de aanwezigheid van CD163 op PVM vinden we ook een aantal andere receptoren op PVM, welke een rol spelen bij de herkenning van mogelijke ziekteverwekkers uit het bloed en bij het op gang brengen van een immuunreactie.

Vervolgens vroegen wij ons af of PVM in staat zijn om de hersenen te verlaten om naar de lymfeklieren in de hals te gaan (Hoofdstuk 5). Het is namelijk bekend dat een afweerreactie pas kan ontstaan als het antigeen (in het geval van MS, myeline) in de lymfeklier aan witte bloedcellen (T-cellen) gepresenteerd wordt. Om dit te onderzoeken hebben we stukjes weefsel van de halsklieren weggenomen bij MS-patiënten en gezonde controles en gekeken of er myeline-afbraakproducten aanwezig waren. Het

blijkt dat er inderdaad myeline eiwitten te vinden zijn in de lymfeklieren van MS-patiënten, wat impliceert dat de halsklieren een belangrijke rol zouden kunnen spelen bij de ontstekingsreactie in de hersenen van MS-patiënten. Hoewel we de myeline eiwitten in de lymfeklieren vonden in macrofagen, durven we niet met zekerheid te zeggen dat de PVM het vanuit de hersenen daarnaar toe gebracht hebben.

3.2. Een rol voor CD163 tijdens MS?

CD163 zit niet alleen op hersenmacrofagen maar ook op de voorloper cellen hiervan, de monocyten, die in het bloed circuleren (Figuur 1). Het was echter onbekend of in het bloed van MS-patiënten een verhoogde expressie van CD163 gevonden kan worden net als in de hersenen (Hoofdstuk 6). Het blijkt dat er bij MS-patiënten minder CD163 op de monocyten zit dan bij gezonde mensen. Wel is er bij mensen met MS meer CD163 los in de circulatie aanwezig (niet-celgebonden). Wat de functie is van dit niet-cel gebonden CD163 is nog niet bekend. Wel weten we dat CD163 van een cel afgeknipt wordt door een knipenzym. De activiteit van dit knipenzym blijkt verhoogd te zijn in het bloed van MS-patiënten.

CD163 kan op het celoppervlak van monocyten in een celkweek verhoogd worden door de cellen te kweken in de aanwezigheid van het bijnierschors hormoon (glucocorticoïden). Mensen met MS krijgen een glucocorticoïdkuur (methylprednisolon) als ze een ernstige verslechtering van de klachten hebben. Deze behandeling remt de ontsteking in de hersenen van de MS-patiënten, waardoor de klachten sneller minder worden. Niet alle mensen met MS reageren even goed op een glucocorticoïdkuur. Het zou goed zijn om van tevoren te kunnen voorspellen of patiënten goed zullen reageren op zo'n kuur.

Wij hebben onderzocht wat er met CD163 op monocyten gebeurt tijdens een glucocorticoïdkuur (Hoofdstuk 7). Het blijkt dat de hoeveelheid CD163 op de cellen aanzienlijk omhoog gaat tijdens een kuur. Dit gebeurde echter niet bij alle patiënten. We vroegen ons daarom af of dit verband hield met de klinische reactie op de behandeling. We hebben aanwijzingen gevonden dat de mate van verhoging van CD163 op monocyten na de behandeling met glucocorticoïden, kan voorspellen of patiënten klinisch op de kuur gaan reageren. Deze bevinding is erg interessant en kan uiteindelijk resulteren in een klinische test om de effectiviteit van een kuur te voorspellen. Dit kan voorkomen dat mensen met MS onnodig een kuur ondergaan. Verder onderzoek met meer patiënten is noodzakelijk voor het zover is.

3.3. De functies van de macrofaag receptor CD163

Nadat we uitvoerig de rol van CD163 in MS onderzocht hebben, was het belangrijk om meer te weten te komen over de functie van deze macrofaagreceptor. Allereerst hebben we gekeken naar de functie van CD163 in de rat en vonden dat deze receptor belangrijk is voor het aanzetten van macrofagen tot de productie van ontstekingsmediatoren (Hoofdstuk 8).

Het was al bekend dat via CD163, hemoglobine kan binden aan macrofagen om vervolgens ontstekingsmediatoren te produceren. Hemoglobuline is een belangrijk onderdeel van rode bloedcellen, dat zorgt voor zuurstoftransport. Naast hemoglobine

hebben we gevonden dat CD163 ook erythroblasten kan binden (Hoofdstuk 9). Erythroblasten zijn voorlopercellen van rode bloedcellen en worden in het beenmerg gevormd. Het blijkt dat CD163 samen met andere receptoren ervoor zorgen dat erythroblasten in een soort van eilandje kunnen uitrijpen tot rode bloedcellen. We hebben laten zien dat onder invloed van CD163 er meer erythroblasten gemaakt worden.

Als laatste hebben we gekeken naar de binding van bacteriën aan CD163 wat vervolgens tot een immuunreactie leidt (Hoofdstuk 10). CD163 is aanwezig op weefselmacrofagen door het hele lichaam. Binding van verschillende soorten bacteriën kan een goed afweermechanisme van het lichaam zijn om bacteriën te herkennen en tijdig een adequate immuunreactie te geven door de productie van ontstekingsmediatoren. Zoals boven beschreven is CD163 op PVM in de hersenen aanwezig, daardoor kan het een rol spelen bij het wegvangen van bacteriën, voordat deze de hersenen 'binnendringen'. Dit is er belangrijk bij het ontstaan van hersenvliesontsteking.

4. Conclusie

We concluderen dat er indicaties zijn voor een belangrijke rol van CD163 op PVM tijdens ontstekingsprocessen. Deze bevinding baant een weg voor nieuwe studies om de bijdrage van deze receptor aan ontstekingsprocessen verder te bestuderen.

Dankwoord

Dit gedeelte is waarschijnlijk het eerste en meest gelezen stuk van dit boekje, hoewel het het laatste is wat ik schrijf (poeh, poeh wat een opluchting!). Dit boekje begon voor mij dik 4 jaar geleden als een abstract idee maar heeft door de vele helpende handen door de jaren heen steeds meer invulling gekregen en ik ben dan ook trots op wat het geworden is. Ik wil daarom beginnen met iedereen te bedanken met wie ik de afgelopen jaren heb samengewerkt, waarvan een aantal hieronder in het bijzonder.

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De leden van mijn leescommissie: prof.dr. Y. van Kooyk, prof.dr. A. van Nieuw Amerongen, prof.dr. P. van der Valk, dr. B. Uitdehaag en dr. F. Verreck. A special thanks for Prof.dr. S.K. Moestrup for taking the time and effort to attend my thesis defence, I hope you enjoy your stay in Amsterdam.

Holger J. Moller, thank you for the countless ELISA's and PCR's you performed for me. I really enjoyed my stay in Denmark with you.

Toon Ligtenberg en Kamran Nazmi van de orale celbiologie, ontzettend bedankt voor het synthetiseren van de peptides gebruikt in hoofdstuk 9 en 10 en het trachten uit te leggen van de wondere voor mij onbegrijpelijke wereld van de biochemie.

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De rest van de MCBI, bedankt voor de goede werksfeer, de samenwerkingen en de gezelligheid!

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Naast het werk zijn er gelukkig ook vrienden die voor de broodnodige afleiding zorgen. Pantertjes en panter-mannen, jullie hebben voor meer dan genoeg gezellige afleiding gezorgd, het wekelijkse eten, dineetjes, barbeques en biertjes. Ik ben benieuwd wat we met zijn allen nog mee gaan maken! Femke en Thijs, lief dat jullie voor mijn promotie even komen overvliegen, laten we er een feestje van maken!
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Mama, .dank je wel voor je steun en vertrouwen tijdens mijn studie en promotie. Onze telefoontjes een aantal keer per week doen mij altijd heel veel goed. Kleine meisjes worden groot....

Bart, liefje, jij hebt van iedereen hierboven genoemd het meeste naast mij gestaan en me geholpen. Je bent een schat, omdat je ontelbare malen naar mijn oefenpraatjes hebt geluisterd en van commentaar hebt voorzien, zonder jou was het me niet gelukt. You are the sunshine of my life!

Kus, Babs

Curriculum Vitae

Babs Odile Fabriek werd geboren op 8 november 1978 te Amsterdam. In 1996 behaalde zij haar VWO-diploma aan het Rijnlands Lyceum te Oegstgeest. In het zelfde jaar begon zij de studie farmacie aan de Universiteit Utrecht. In 1997 maakte ze de overstap naar de studie medische biologie, tevens aan de Universiteit Utrecht. Tijdens de doctoraalfase van haar studie verrichtte ze onderzoek naar de werking van antipsychotica in een diermodel voor schizophrenie bij het Rudolp Magnus Instituut voor Neurowetenschappen in Utrecht onder leiding van prof.dr. V. Wiegant. Haar tweede wetenschappelijke stage liep zij op het Nederlands Instituut voor Hersenonderzoek te Amsterdam bij prof.dr. D. Swaab waar ze onderzoek verrichte naar de expressie van schildklierhormonen in de hersenen. Na het behalen van haar doctoraalexamen in 2002 is ze in november van dat jaar in dienst getreden als promovendus bij de afdeling moleculaire celbiologie en immunologie van het VU Medisch Centrum te Amsterdam. Onder leiding van prof.dr. C.D.Dijkstra en dr. T.K. van den Berg verrichtte zij een door de Stichting MS Research gefinancierd onderzoek naar de rol van perivasculaire macrofagen tijdens ontstekingen in de hersenen. De bevindingen van dit onderzoek staan beschreven in dit proefschrift.

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